THE CATALYTIC ACTIVITY OF PEPSIN C.

by

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This thesis has been composed by myself and the work presented is my own unless otherwise acknowledged.

SUMMARY

A series of small peptides have been synthesised and used to investigate the activity of a minor pepsin, porcine pepsin C, and to compare the activities of pepsins A and C from man and pig. The peptides had the general form A-Leu-Val-His-B. Hydrolysis by pepsin C at pH 2.07 and 37°C increased as A was ac-Ala, ac-Tyr, ac-Phe and ac-Ala-Phe.B was either the methyl ester, amide or the free acid of histidine, and hydrolysis was exclusively at the N-terminal side of leucine. The ac-Ala peptides were resistant to the hydrolysis by the other enzymes under the conditions used, and the tyrosine peptides were only very slowly hydrolysed by both pepsins A. Studies with the peptide ac-Phe-Leu-Val-His-amide with pepsin C at a range of pH values have led to the estimation of the values of pK of two catalytically important groups as 1.42 and 4.88, similar to those previously observed for pepsin. Inhibition of the hydrolysis at pH 3.01 by acetyl phenyl alanine shows a form of non-competitive inhibition.

Hydrolysis of the peptides ac-Tyr-Leu-Val-His-methyl ester and the corresponding amide by pepsin C show non-classical kinetics which have been discussed in terms of a substrate activating mechanism.

Comparative studies with porcine and human pepsins A and C show the similar specificities, but in general lower activities of the human enzymes. These studies have led to the development of a differential assay for the human enzymes which may be useful in clinical studies.

The kinetic results with porcine pepsin C have been discussed with reference to similar observations made with the major pepsin.

INDEX

SECTION 3. METHODS USED IN PEPTIDE SYNTHESES

SECTION 4. PEPTIDE SYNTHESES

SECTION 6. KINETIC STUDIES WITH PEPSIN C

N.B. Abbreviations which are used in the text are those recommended by I.U.P.A.C.

LIST OF TABLES

LIST OF FIGURES

SECTION 1 INTRODUCTION

1.1 Historical background

Some of the earliest recorded evidence that the digestion of meat was a chemical rather than a mechanical process was given by Reamur in the early eighteenth century. He obtained some gastric juice, from a pet bird, which was capable of partial digestion of meat outside the body. The work was furthered by Spallanzani (1783) working with human gastric juice and later, (Schwann 1836), it was proposed that gastric juice, which was shown to be very acid, also contained a neutral component, which in the presence of acid, was active in the partial digestion of meat. The name pepsin (from the Greek, pepsis = digestion) was given to this component. Towards the end of the nineteenth century, attempts were made to isolate pepsin and it was also at this time that Langley (1881) recognised the existence of an alkali stable precursor to pepsin, which could be converted to pepsin by the addition of acid; Kühne gave the name enzyme to the biological catalysts and it was also suggested at the end of the nineteenth century that the activity might be associated with proteins. This proposal, however, was not widely accepted until the work of Sumner (1926) with urease and Northrop (1930) with pepsin, showed that enzyme activity was associated with purified proteins.

1.2 Distribution and heterogeneity of pepsins

Pepsins have been found in all mammals, birds and fish examined, and enzymes showing similar activity at acid pH values have also been isolated from fungi. Porcine pepsin, which has been used for most of the enzymological studies, was crystallised by Northrop (1930).

Pepsins have also been crystallised, for example from ox, (Northrop 1933), man (Tang et al. 1959), chicken (Bohak 1969), salmon (Norris & Elam 1940) and smooth dog fish (Merrett et al 1969). The extra cellular acid proteases from Penicillium janthinellum (Sodek & Hofmann 1968), Rhizopus chinensis (Fukumoto et al. 1967) and Endothia parasitica (Sardinas 1968) are among those which have been studied from fungi. Chymosin (formerly rennin), the milk clotting enzyme of calves, which is related to pepsin, was crystallised by Berridge (1945) and interest in many of the fungal proteases has arisen due to their potential use as alternatives to chymosin in the cheesemaking industry. Acid proteases are also reported to have been isolated from two higher plants (Hofmann 1974) occurring in the seeds of the Lotus and the secretion from the pitcher plant (Nepenthes sp).

The heterogeneity of porcine pepsin, which was initially isolated by extraction of the gastric mucosa with acid, was suggested by several authors. Takemura (1909) and Hirayama (1910), suggested there was more than one proteolytic enzyme, because the crude extract showed a higher activity at pH 4 than did the purified preparations. Michaelis and Davidsohn (1910) using electrophoresis, implied the presence of more than one protein component in their pepsin preparation. pH dependence curves of the activity of gastric juice and purified pepsins in the digestion of certain proteins showed two peaks of activity. For example Dyckerhof and Tewes (1933) showed a peak at pH 2 and one at pH 3.5-4 in the digestion of casein, gelatin and edestin by porcine pepsin, and Buchs (1947) demonstrated two similar maxima when human gastric juice acted on edestin. The action of pepsin on egg albumen shows only a single peak at about pH 2 (Taylor 1959 and earlier references cited therein). It was suggested that two pH activity peaks might represent the action of more than one

enzyme, but Taylor (1968 for a review) pointed out that this might also be caused by a single enzyme acting differently at two substrate sites, or by the presence of two active sites on the enzyme molecule. Even the crystalline preparations appeared unhomogeneous by their pattern of solubility (Desreux & Herriott 1939) and electrophoretic properties (Hoch 1950), while during the purification procedure an amorphous precipitate, which was extremely active in liquefying gelatin, was formed (Northrop 1931). Ryle and Porter (1959) isolated two minor pepsins now called pepsins B and C by chromatography on DEAE cellulose, and subsequently a third minor pepsin, pepsin D, which is a non-phosphorylated pepsin A, and all the corresponding zymogens have been isolated (Ryle, 1960a, 1965; Lee & Ryle 1963). The system of nomenclature of the pepsins as described above has been recommended by the International Union of Pure and Applied Chemistry 1972 and the International Union of Biochemistry (nos. 3. 4. 23).

Paper chromatography of human gastric juice by Caputto et al. (1954) showed the presence of two enzymes, whilst other workers, using various media, have proposed the existence of at least four gastric enzymes and their zymogens, (e.g. Kushner et al. 1964; Seijffers et al. 1963 a & b; Hanley et al. 1966). Richmond et al. (1958) have isolated two of the human enzymes from gastric juice, these being pepsin and gastricsin, which has similar properties to porcine pepsin C. Despite the proposed existence of more human gastric enzymes, and their zymogens, they have not been isolated and characterised in quantity. The nomenclature of these enzymes and their zymogens is confused, as they have generally been named according to the pattern of elution from ion exchange media, or the mobility in electrophoresis in the system which has been used. Taylor (1968) has attempted to rationalise

Figure 1 Comparison of the N-terminal sequences of various "Pepsins"

(Adapted from Sepulveda *et al.* 1975)

Human Pepsin	VAL-Asp-Glu-Gln-PRO-Leu-Glu-ASN-TYR-Leu - ASP-Met-GLU-TYR-PHE-GLY
Porcine Pepsin	Ala-Leu-Ile-Gly-Asp-Glu-PRO-Leu-Glu-ASN-TYR-Leu - ASP-Met-GLU-TYR-PHE-GLY
Bovine Pepsin	VAL-Ser-Gln-Glu-PRO-Leu-Gln-ASN-TYR-
Gastricsin	$\frac{\text{Val}}{\text{Ser}}$-VAL-Thr-Tyr-Glu-PRO-Met-Ala - TYR-Met - ASP-Ala-Ala-TYR-PHE-GLY
Bovine Chymosin	Gly-Glu-VAL-Ala-Ser-Val-PRO-Leu-Thr-ASN-TYR-Leu - ASP-Ser-Gln-TYR-PHE-GLY
R. chinensis acid protease	Ala-Gly-VAL-Gly-Thr-Val-PRO-Met-Thr-ASP-TYR-Gly-Asn-ASP - GLU-TYR-Tyr-GLY
Penicillopepsin	Ala-Ala-Ser-Gly-Val-Ala-Thr-Asn-Thr-Pro-Thr-Ala-Asn-ASP-Glu-GLU-TYR-Ile-Thr
Human Pepsin	Thr-ILE-Gly-ILE-GLY-THR-PRO-Ala-Gln-Asp-PHE-THR-VAL-Val-PHE-ASP-THR-GLY-
Porcine Pepsin	Thr-ILE-Gly-ILE-GLY-THR-PRO-Ala-Gln-Asp-PHE-THR-VAL-Ile-PHE-ASP-THR-GLY-
Gastricsin	Glu-ILE-Ser-ILE-GLY-THR-PRO-Ala-
Chymosin	Lys-ILE-Tyr-Leu-GLY-THR-PRO-Pro-Gln-Glu-PHE-THR-VAL-Leu-PHE-ASP-THR-GLY-
R. chinensis acid protease	Pro-VAL-Thr-ILE-GLY-THR-PRO-Gly-
Penicillopepsin	Pro-VAL-Thr-ILE-GLY-Gly-Thr-Thr-Leu-

the nomenclature using the electrophoresis of gastric juice in Agar gels as a basis, and Whitecross et al (1974) have used a similar system, achieving slightly better resolution and correlating their results with DEAE cellulose ion exchange chromatography. Samloff (1969) and Turner et al (1970) have developed a similar method for the zymogens.

Some studies have been made on the structural homologies of the various pepsins and their zymogens. The total sequence of porcine pepsin and its zymogen have been reported (Tang et al 1973; Sepulveda et al 1975b, Moravek & Kostka 1974). The molecule consists of a single polypeptide chain, and has a very high ratio of acidic to basic residues, there being about 40 aspartic and glutamic acids but only four basic amino acids. There are three disulphide bridges. The amino and carboxy terminus sequences of several other acid proteases are known. For example, Sepulveda et al (1975a) have illustrated the homologies between various acid proteases, their information being taken from several sources, for the amino terminal regions. A similar figure is shown opposite (Figure 1). Human and porcine pepsin A are almost identical over the first thirty or so residues, although one form of porcine pepsin has an additional two residues at its amino terminus. Gastricsin also shows a high degree of homology with pepsin A as does bovine chymosin. The carboxy-termini also show a marked homology (Huang & Tang 1970). Of the two fungal proteases shown, that from Rhizopus chinensis shows a closer degree of relatedness than does penicillopepsin over this region, although according to Hofmann (1974) penicillopepsin shows a high degree of homology with the gastric proteases at the carboxy-terminus. From the data so far available it seems likely that gene

doubling gave rise to the separate gastric enzymes, pepsin and gastricsin, which perhaps took place before species divergence.

1.3 General properties of pepsins

Pepsins are formed in the gastric mucosal cells as their zymogens, which become activated at acid pH to the enzyme. The activation procedure involves the removal of about forty residues, including most of the basic residues, from the amino terminus of the zymogen. This is believed to be an intramolecular activation below pH 3, and intermolecular between pH 3 and pH 5 (Al-Janabi et al 1972). The process is thought to proceed by more than one step, (Dykes and Kay 1976). These authors found that the first pepsin-like compound, which could be inhibited by pepstatin during the activation process of porcine pepsinogen, consisted of all but the first sixteen residues of the zymogen. The final activation mixture in the bovine system consists of the active enzyme and several peptides, one of which (Harboe et al 1974) acts as an inhibitor to the milk clotting activity of the enzyme. This is believed to be the residues 1-17, which are homologous to the porcine zymogen.

The pepsins have similar enzymic activities, although some slight variations do occur. Pepsin C and gastricsin, for example, are unable to hydrolyse the synthetic peptide acetyl-L-phenylalanyl-L-diiodotyrosine, which is a good substrate for pepsin A. Pepsin B however has a high specific activity towards this peptide but only has low haemoglobin digesting activity. Although pepsin A from pig and human sources is unstable at pH 6.9, chicken pepsin is quite stable at pH values below 8 (Bohak, 1969). Chicken pepsin is also thought to have some carbohydrate associated with it, which has not

been detected in enzymes from human and porcine sources. Porcine pepsin A is phosphorylated at a serine residue which is number 68 in the total sequence, but the phosphate is not essential for activity (Perlmann 1955).

Viscosity and sedimentation data suggest that pepsin is compact and nearly spherical. Optical rotatory dispersion studies indicate that there is very little alpha helical content in the enzyme (Jirgensons 1958; Perlmann 1959). X-ray studies with pepsin have been reported by Andreeva et al (1971) (and cited in Fruton 1976) and for Endothia parasitica acid protease by Jenkins et al (1975). They show an ellipsoidal molecule about 600 nm x 400 nm x 350 nm with a cleft of approximately 250 nm which they suggest might represent the active site region.

Estimates for the molecular weight of pepsin have varied from 23,900 - 37,600, the commonly accepted value being 34,500. Examples of the values obtained by different methods are cited in Herriott (1962) and Williams and Rajagopalan (1966). Wide variations have also been reported for the molecular weight of pepsinogen.

1.4 Introduction to kinetic studies

Early studies on the rates of enzyme catalysed reactions led to the proposal for the existence of an intermediate in the reaction pathway which then broke down to give products. It was pointed out by Henri (1902, 1905) that this would explain the observation that the rate of decomposition of substrate reached a constant value when the substrate concentration was increased far enough. This is termed the Maximum velocity (Vmax) at a given enzyme concentration. This was expressed quantitatively by Michaelis and Menten (1913) and a

current representation for the most simple case

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

where E represents enzyme, S and P are substrate and products respectively and k_1, k_{-1}, k_{cat} are the rate constants for the steps, is $v = \frac{k_{cat}\ E_t\ S_o}{Km + S_o}$, where v represents the initial velocity, E_t is total enzyme concentration, S_o is the initial substrate concentration and Km, the Michaelis constant $= \frac{k_{cat} + k_{-1}}{k_1}$. When the equilibrium between enzyme and substrate is very rapid in comparison with the rate of breakdown of the complex , i.e. when $k_{cat} \ll k_{-1}$, Km approximates to Ks, the dissociation constant of the complex. Thus the value of Km is often regarded as an inverse measure of the tightness of binding of the substrate to the enzyme.

The parameter $\frac{k_{cat}}{Km}$ is often used as a measure of the efficiency of an enzyme towards a given substrate, and it can be measured quite accurately even when the individual values of Km and k_{cat} cannot be determined, for example when Km is larger than the levels of substrate that can be used. $\frac{k_{cat}}{Km}$ approaches a first order rate constant as S_o approaches zero, and therefore it may be estimated from the tangent at zero substrate concentration in a plot of initial velocity versus substrate concentration. This gives a value of $\frac{Vmax}{Km}$, and division by total enzyme concentration gives the required ratio.

It was realised that kinetic studies on the action of enzymes with their substrates could give useful information which might lead to an understanding of the mechanism by which these catalysts worked. However, detailed kinetic work with pepsin acting on protein substrates is not possible, since several bonds are

cleaved in the hydrolysis and at presumably different rates. Hence synthetic peptide substrates were developed and used, leading to information about the catalytic groups in the active site, by pH dependence studies, and also about the specificity of the enzyme towards the amino acid residues on either side of the bond to be cleaved.

The first synthetic substrates for pepsin, Z-Glu-Tyr, and Z-Gly-Tyr-amide were introduced by Fruton and Bergmann (1938, 1939). These, however, were extremely insoluble in the pH range of peptic activity (1 - 5) and were cleaved extremely slowly. This, and other early work, Fruton, Bergmann and Anslow (1939); Harington and Pittrivers (1948); Dekker et al (1949) seemed to show a requirement for amino acids of an L-configuration and also that a phenylalanyl or tyrosyl residue had to provide the NH group to the peptide bond which was cleaved. Progress was made by Baker (1951) who used acetyl di-peptides such as ac-Phe-Tyr which were cleaved much more rapidly than Z-Glu-Tyr, and her work indicated the preference for aromatic residues on both sides of the susceptible bond. The best synthetic substrate known for many years for pepsin was acetyl-L-phenylalanyl-L-diiodotyrosine, Km being lower and k_{cat} higher than for the parent compound ac-Phe-Tyr.

Subsequent studies on bonds cleaved during the peptic hydrolysis of proteins have shown pepsin to have a fairly wide side chain specificity, with a preference for aromatic residues or hydrophobic residues such as leucine, (Tang 1963; Hill 1965).

Much work has now been done to try to define the specificity of pepsin action, as measured by the hydrolysis of synthetic peptides.

Series of peptides whose character is systematically changed by altering a single residue or by extending the peptide chain in either direction, have been developed and the effect on peptic hydrolysis measured.

Peptides have generally been of the type A-X-Y-B, where the X-Y bond is the one to be cleaved. The group A is generally an acylating group such as benzyloxycarbonyl or acetyl and B an ester or simply the free carboxyl group of the amino acid. In some cases A and B represent an extension of the peptide chain with additional amino acid residues.

Fruton and co-workers wished to avoid the presence of free carboxyl groups in the substrates, especially on the residue adjacent to the bond to be cleaved, since the pKa of the group falls within the range of pH to be studied. It has been found that a carboxyl group in this position can inhibit the hydrolysis of a sensitive bond, as illustrated by the rates of hydrolyses of the peptide Z-His-Phe-Phe and its corresponding methyl ester (Inouye et al 1966). The optimum pH for hydrolysis of the ester is pH 4 while that for the free acid is pH 3. It was considered that the carboxylate group would be largely protonated at pH 3 and would thus no longer exert its inhibiting effect.

In order to increase the solubility of the peptides, a cationic group such as a histidine residue or a pyridyl ring in the ester blocking group was introduced. However, the possible effect of a charged group in the vicinity of the sensitive bond must be borne in mind when interpreting results, especially in the higher regions of the pH range 1 - 6 where, for example, the

ionisation of a histidine residue may become involved (Hollands & Fruton 1968). Some workers have therefore preferred the use of neutral aromatic peptides (e.g. Jackson et al 1965; Zeffren & Kaiser 1966; Denburg et al 1968; Clement et al 1968).

Many results have been obtained using peptides as described above in investigations of the specificity of action of pepsin, and the effects of pH, inhibitors and isotopes on the hydrolytic reaction. Several reviews have been published describing these observations in detail, and leading to various proposals for the mechanism of pepsin action (Fruton 1970, 1976; Knowles 1970; Clement 1973; Hofmann 1974).

For small synthetic substrates of the type A-X-YB as described above, kinetic studies have shown both X and Y are involved in binding to the active site which is hydrophobic (Silver et al 1965; Jackson et al 1965, 1966; Schlamowitz et al 1968). Phenylalanine is preferred for the X position and tryptophan, phenylalanine or tyrosine is preferred at Y.

Fruton (1970) has shown that a tyrosyl residue at X seems to inhibit the reaction, although if the phenolic hydroxyl is masked with a methyl group, the inhibiting effect is partly overcome. Also, the planar character of an aromatic group, or an unbranched carbon in an aliphatic residue at X seems to be important in the interaction of enzyme and substrate since a β-cyclohexyl-L-alanyl residue or β-carbon branched aliphatic residue reduces the rate of hydrolysis. Throughout the experiments, very little variation is seen in the values of Km for the different substrates, the effect of increased "efficiency" mainly being due to an increase

TABLE 1

Kinetic parameters for the hydrolysis of synthetic peptides by pepsin A (adapted from Fruton 1976)

Substrate	kcat(s^{-1})	Km(mM)	$\frac{kcat}{Km}$($s^{-1}mM^{-1}$)	Ref
Z-Phe-Phe-OP4P*	0.7	0.2	3.5	(1)
Z-Gly-Ala-Phe-Phe-OP4P	409	0.1	4090	(2)
Z-Ala-Ala-Phe-Phe-OP4P	282	0.04	7050	(1)
Z-His-Phe(NO_2)-Phe-OMe	0.26	0.43	0.6	(3)
Phe-Gly-His-Phe(NO_2)-Phe-Val-Leu-OMe	62	0.04	1540	(4)

1. Sachdev and Fruton, 1969.
2. Sachdev and Fruton, 1970.
3. Voynick and Fruton, 1971.
4. Ferguson, Andrews, Voynick and Fruton, 1973.

* OP4P - 3-(4-pyridyl) propoxy.

in k_{cat}.

Km has been shown to approximate to Ks for pepsin catalysed reactions by competitive inhibition studies (e.g. Zeffren & Kaiser 1968; Inouye & Fruton 1968; Denburg et al 1968; Knowles et al 1969) and by using fluorometric studies of substrate binding (Sachdev et al 1972; Sachdev et al 1973, 1975).

For the peptides Z-Phe-X, where X is diiodotyrosine, dibromotyrosine or tyrosine and assuming $Km \simeq Ks$, Zeffren and Kaiser (1966) showed that the diiodo compound bound more readily than the dibromo one, and this more than the tyrosine itself. They interpreted this as possibly being due to better fit in the active site, or due to an electrostatic interaction becoming involved between the enzyme and the increasingly polarisable halogen electrons. A further significant result which emerged from the studies with A-X-Y-B peptides by Fruton and his colleagues is the effect of extending the peptide chain to either side of the susceptible bond. The major features of this study are that the values of Km remain within one order of magnitude, whilst k_{cat} may alter up to a thousand fold. The most extreme examples are for peptides tested at pH 3.5 - 4, shown in Table 1., adapted from Fruton (1976). It should be noted however that although most extended peptides showed an improvement in $\frac{k_{cat}}{Km}$, not all of them did, e.g. Z-Gly-Pro-Phe-Phe-OP4P showed a tenfold decrease in $\frac{k_{cat}}{Km}$ compared with the parent compound Z-Phe-Phe-OP4P. Overall, these results have led to the proposal for important secondary binding sites in the interaction between substrates and pepsin at points distant from the active site. Despite the fact that a low Km, implying tight binding at the active site, is considered a good feature for a substrate, examples have

been found where a substantial increase in Km does not exclude an increased rate of hydrolysis, implying that tighter binding is not necessary for increased hydrolysis. Fruton has also observed (1970) that the actual position of the histidine residue in his cationic substrates can affect the susceptibility of a given bond, suggesting that perhaps there is a special interaction between the enzyme and a histidine occurring next to the susceptible bond. For example $\frac{k_{cat}}{Km}$ values for Z-His-Phe-Phe-OEt, Z-Gly-His-Phe-Phe-OEt and Z-His-Gly-Phe-Phe-OEt, hydrolysed at the Phe-Phe bond, are 2.6, 7.7 and 0.2 s^{-1} mM^{-1} respectively at pH 4.5.

Many workers have reported the influence of pH on the kinetic parameters of synthetic peptides when hydrolysed by pepsin. Bell shaped curves were obtained for the dependence of k_{cat} or $\frac{k_{cat}}{Km}$ with pH with various substrates (e.g. Denburg et al 1968; Clement et al 1968; Cornish-Bowden & Knowles 1969) suggesting two catalytically important groups on the enzyme substrate complex or free enzyme respectively, when testing neutral substrates. pK values of about 1 and 4.5 were obtained depending slightly on the substrate used. Substrates with a free carboxyl group showed a pK in the region of 3.5, reflecting the pKa of the substrate. Jackson et al (1969) suggested that a group on the enzyme with a pK of about 4 must be protonated for substrate to be bound, and also, a group with a pK on the ES complex of about 4.1 if a carboxylate group is present on the substrate, or 4.7 if it is absent, which must be protonated for hydrolysis. This data is hard to reconcile with that of other workers. Also they do not seem to have taken account of the low pH range of their data, which if extended a little, look as though they might show an important pK in the region of pH 1. Hunkapiller and Richards

(1972), studying the hydrolysis of trifluoroacetyl amino acids by nmr, reported catalytically important groups on the enzyme with pKa values of 2.8 and 3.7. A further group which they suggested to be important in binding had a pK of about 4.7. These values do not agree with those of other workers, although the pK which was shown as 3.7 may reflect the pKa of the substrate rather than the enzyme, the value of 2.8 is very much higher than other estimations of the group with a low pKa.

In studies with cationic substrates, a slight shift in the pK values has been observed (Hollands & Fruton 1968) as described earlier, and this is perhaps a drawback to the use of cationic substrates.

It was suggested that two carboxylate groups were present in the active centre of pepsin, one being ionised and the other protonated in the normal range of activity of the enzyme. This has led to many studies of chemical modification of the enzyme, giving more direct evidence for the involvement of two carboxyl groups. These studies will be discussed later.

Proposals have been made for the mechanism by which pepsin exerts its catalytic effect, and it has generally been considered necessary for a covalent intermediate to be formed, which is then hydrolysed to give products. Fruton (1976) and Hofmann (1974) have pointed out that strong non-covalent forces could be involved. The three pathways which have been proposed are: first, the formation of an amino-enzyme intermediate, that is the amino portion of the susceptible bond becomes covalently attached to the enzyme, with the simultaneous release of the carboxylic acid portion. Hydrolysis

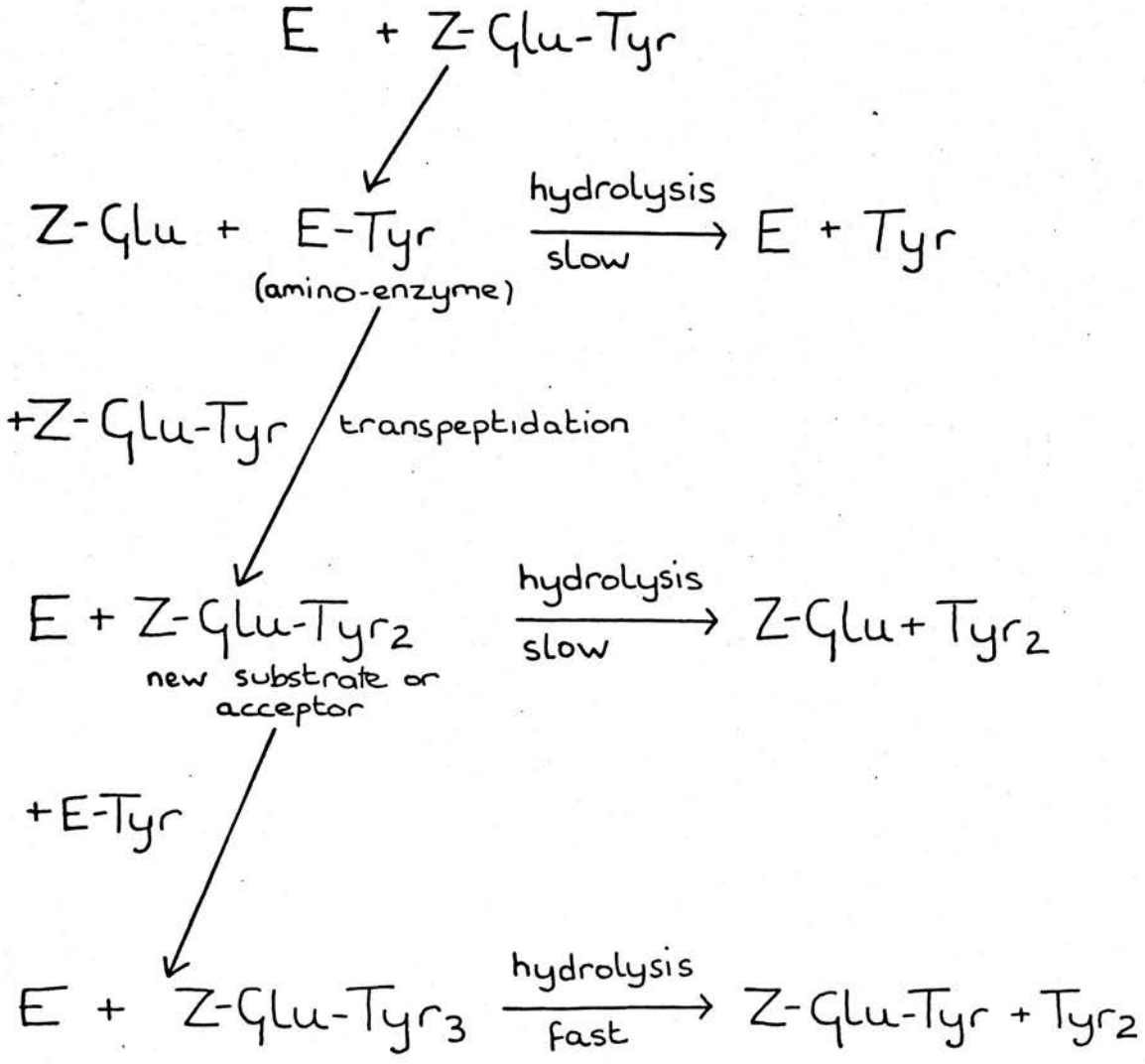


FIGURE 2. Scheme for pepsin catalysed transpeptidation, adapted from Neumann et al. (1959.)

of the amino enzyme then releases the amine and regenerates the free enzyme (Knowles 1970; Fruton 1970). Secondly, the formation of an acyl enzyme intermediate with the simultaneous release of the amine moiety, and subsequent hydrolysis of the acyl enzyme (Hofmann 1974). Thirdly, the formation of a double enzyme intermediate, one of the carboxyl groups bearing the amino portion in an amide bond, and the other the carboxyl portion in an acyl bond. Hydrolysis would yield products and regenerate the free enzyme (Bender & Kezdy 1965; Clement & Snyder 1966; Clement 1973).

Evidence has been put forward in support of and against each proposal, and the most recent reports (Hofmann 1974; Fruton 1976) suggest that there may not be a single unique mechanism of action.

The ability of pepsin to catalyse transpeptidation, when reacting with small peptides, was observed by Neumann et al. (1959). This was apparent only at fairly high pH values and for quite poor substrates for the hydrolysis reaction. The results obtained by Neumann et al., and also, for example, Kitson and Knowles (1971b), Silver and Stoddard (1972), were consistent with the formation of an amino-enzyme intermediate, the amino portion then being accepted by a carboxyl group rather than water.

For example in the reaction of pepsin with Z-Glu-Tyr, both hydrolysis products, Z-Glu and Tyr were observed, together with the dipeptide Tyr_2. Thus Z-Glu-Tyr accepted Tyrosine from an amino enzyme, the product could then be hydrolysed to give tyrosyltyrosine or act as another acceptor which is then hydrolysed and so on. A scheme is shown in figure 2 for the reaction. Takahashi and Hofmann (1972) have demonstrated, that a related acid protease to porcine

pepsin i.e. penicillopepsin would catalyse transfer reactions consistent with an acyl intermediate, and this has been further studied for pepsin (Takahashi & Hofmann 1975; Wang & Hofmann 1976a; Newmark & Knowles 1975). Prior to this work, the evidence for the existence of an acyl enzyme intermediate had been poor. The ability of pepsin to catalyse O^{18} exchange between $H_2{}^{18}O$ and virtual substrates such as Z-Phe was thought to be consistent with an acyl enzyme intermediate (Sharon, Grisaro & Neumann 1962). However an alternative explanation is that one of the carboxyl groups in the active site of pepsin can incorporate ^{18}O (Shkarenkova et al. 1968) which is then transferred to Z-Phe by a concerted mechanism, no enzyme intermediate being formed.

Despite the implications of transpeptidation studies it has not been possible to trap an acyl intermediate using C_{14} methanol, which has been found suitable in studies with neutral proteases (Cornish-Bowden et al. 1969).

The formation of an enzyme intermediate with simultaneous release of one product implies that the products are released in order. Under suitable conditions of high enzyme concentration, and with the substrate concentration being both greatly in excess of the Km and enzyme concentration, a 'burst' release of product should be obtained prior to the establishment of the steady state. Inouye and Fruton (1967) spectrophotometrically monitoring the release of Z-His-Phe($4NO_2$), from Z-His-Phe($4NO_2$)-Phe-OMe observed no sudden release of the acid, (also Cornish-Bowden et al. 1969). Thus if an amino enzyme occurs it must do so after the rate limiting step. Sachdev and Fruton (1975), using stopped flow techniques with peptides bearing a fluorescent label, found no direct evidence

for ordered release of products, both appearing to leave the active site at the same time.

Kinetic evidence for the ordered release of products, the carboxyl group leaving first, was presented by Kitson and Knowles (1971a). The linear non-competitive inhibition of Ac-Phe, and other product analogues at pH2 in the hydrolysis of Ac-Phe-Phe-Gly was said to be consistent with acetyl-phenylalanine being released first (after Cleland 1963). However at pH 4.3 Ac-Phe weakly inhibits the hydrolysis in a competitive manner (Kitson & Knowles 1971a). Silver and Stoddard (1975) have suggested the alternative explanation, that the inhibitor complex forms before the rate limiting step, which would also give rise to non-competitive inhibition but does not imply ordered product release. The pattern of non-competitive inhibition was not consistent with the formation of a unique amino-enzyme. The release of products may depend on the actual structure of both the carboxyl and amino portions of the peptide. The apparent absence of a unique amino enzyme was also suggested by the results of some transpeptidation experiments (Silver & Kelleher - reported in Fruton 1976, ref 99a), although this is in contrast to earlier work of Antonov et al. 1974. The group A, in the peptide A-Phe($4NO_2$)-Tyr, when A is Z, Z-Gly or Z-Gly-Gly, affects the rate at which Tyrosine leaves the active site. This may be considered as evidence for the secondary binding effects causing a conformational change at the active site, which not only affects the rate of the hydrolysis, but also influences the release of products. The nature of both leaving groups could thus affect the nature of a covalent intermediate, if formed at all, and also the release of products from the active site. The fact that the

transpeptidation experiments, with poor substrates are the only ones which can now be said to consistently infer the ordered release of products shows that the mechanism for the action of pepsin on good peptide substrates, far less proteins, is uncertain so far.

Most of the proposals for a pathway leading to a covalent enzyme intermediate begin with the attack by the ionised carboxyl group at the active site on the electron deficient carbon of the susceptible peptide bond in the substrate. This leads to a tetrahedral intermediate, and Knowles (1970) and Fruton (1970) suggest the process is assisted by hydrogen bonding with the second, protonated, carboxyl group. The proposal by Bender and Kezdy (1965) that reversible formation of an anhydride takes place at the active site, and then reacts with the substrate is not well supported in organic chemistry. For the formation of an amino-enzyme, Knowles (1970) and Fruton (1970) have proposed a four-centre exchange of the tetrahedral intermediate, the amino group attaching to the carbon of the carboxyl group which initially attacked the peptide bond. The carboxylic acid moiety is generated at the same time and may be released. However the four-membered ring implied in the transition state of the transfer is not well supported in organic chemistry and would involve highly strained bond angles. The mechanism proposed by Hunkapiller and Richards (1972) involves two carboxyl groups in the formation of the tetrahedral intermediate, and a third which is involved in its rearrangement. According to the proposal of Clement (1973) the tetrahedral intermediate converts to the double intermediate. For the amino enzyme to form, as is suggested by the transpeptidation studies, hydrolysis with water would release $R\text{-}COO^-$ leaving the required

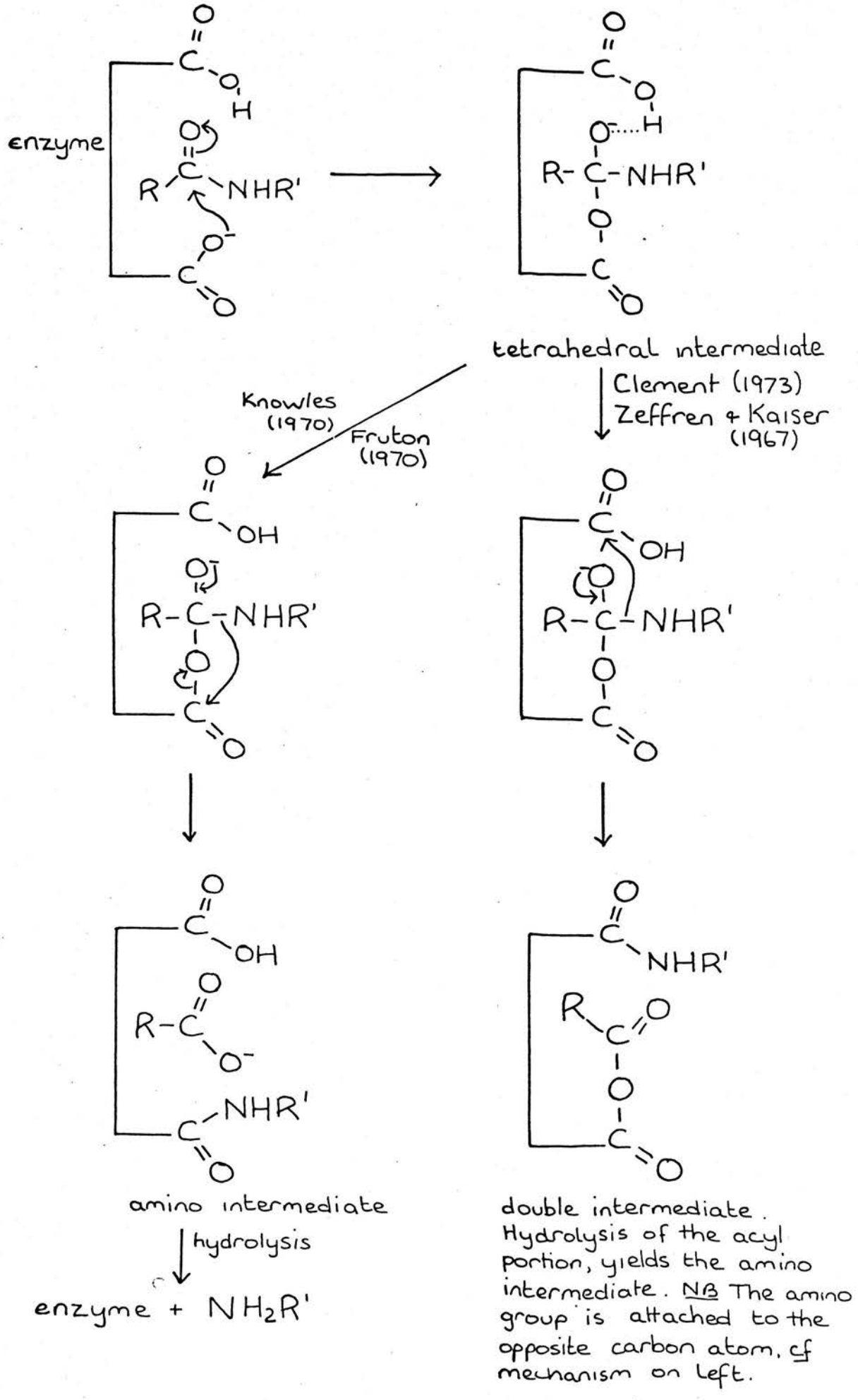


FIGURE 3. Proposed mechanisms of pepsin action.

enzyme intermediate. Note that the nitrogen is bonded to the opposite carboxyl group to that proposed by Knowles and Fruton. This is illustrated in the representation of the mechanism in figure 3. Scheme I of Zeffren and Kaiser (1967) has a similar implied mechanism. The amino-enzyme may be cleaved with the assistance of the adjacent carboxyl group as suggested by Clement (1973), Knowles (1970) and Delpierre and Fruton (1965). Using the hydrolysis of phthalamic acid as a model in organic chemistry, this proceeds via an anhydride intermediate, which is very rapidly hydrolysed to regenerate the free enzyme. These methods were all proposed when the amino-enzyme was more favoured on the pathway of hydrolysis. The pathways described could be extended to include the formation of an acyl intermediate if the nitrogen donates electrons to a hydrogen (hydronium) ion. Recently Fruton (1976) has suggested the use of dialkylmaleamic acid as a chemical model for producing an acyl enzyme. For the formation of an acyl intermediate, the double intermediate would be less likely, as the amide bond of the intermediate would need to be broken while the more labile acyl enzyme remained intact. The assistance of a third catalytic group would probably need to be involved and even then the pathway seems a little unlikely.

Hofmann (1974) suggested that either, but not both, enzyme intermediates could be formed, depending on the substrate. Different pathways would be involved perhaps with different rate limiting steps, which may explain why deuterium isotope kinetic effects have been found for some substates, for example Gly-Gly-Gly-Phe($4NO_2$)-Phe-OMe (Hollands & Fruton 1969), but not with others, e.g. Ac-Phe-Tyr-OMe (Bender & Kezdy, 1965; Clement & Snyder 1966).

Thus the mechanism of pepsin action is highly complex and at present not completely understood.

1.5 Chemical modification of pepsin

Early work on the chemical inhibition of pepsin by bis-(β,β' chloroethyl) sulphide - mustard gas (Herriott et al. 1946) and the early kinetic studies on the hydrolysis of peptides at various pH values suggested that carboxyl groups might be involved in the catalysis. Similarly, the work of Herriott (1947) and Delpierre and Fruton (1965) with diazomethane and diphenyl diazomethane implied the involvement of one or more catalytically important carboxyl groups. Many reagents were developed such as diazoacetyl amino acid esters, diazoketones, and various derivatives (e.g. Bayliss et al. 1969; Delpierre & Fruton 1966; Ong and Perlmann 1967). The diazoacetyl amino acid esters generally had the amino acid as an 'active site directed' moiety, for example, leucyl or phenylalanyl (e.g. Rajagopalan et al. 1966b and Delpierre & Fruton 1966). However, Lundblad and Stein (1969) said that the stoichiometric inactivation of pepsin did not depend on the side chain amino acid, since the glycyl derivative also caused total inhibition of the catalysis. Copper ions were required for the inactivation, and a single aspartic acid residue became labelled. This was found to be in the sequence Ile-Val-Asp-Thr- (Fry et al. 1968). An extended sequence was reported by Bayliss et al. (1969), and the aspartic acid residue has now been assigned to residue 215 in the total sequence. The chemistry of the inactivation implies a high pKa (>4) for the group.

Hartsuck and Tang (1972), using 1,2-epoxy-3-p-nitrophenoxy propane at 4°C found an aspartic acid in the sequence Ile-Phe-Asp-

Thr-Gly-Ser-Ser-Asn-, and this is now assigned to residue 32 in the total sequence. Stoichiometric proportions of inhibitor were incorporated up to 80% inactivation towards bovine haemoglobin as substrate, suggesting active site modification, but then a methionine residue also became modified. A very low pK of the carboxyl group is suggested by this modification, in agreement with the kinetic predictions.

A further aspartic acid was implicated by its specific reaction with p-bromophenacyl bromide (Erlanger et al. 1965) in stoichiometric proportions. However total inactivation did not result, and it was suggested that the residue in the enzyme was near the active site, and its modification sterically inhibited the approach of substrate.

A study by Paterson and Knowles (1972) using the non specific modifier of carboxyl groups, trimethyloxonium fluoroborate and analysing their data by the method of Tsou Chen-Lu (1962), suggested a minimum of two carboxyl groups are necessary for catalysis.

Modification of other residues has also been studied. In 1935, Herriott studied the effect of tyrosine modification. Lokshina and Orekhovich (1966) and Perlmann (1966), have found that tyrosine modification leads to decreased activity towards haemoglobin but increased activity towards certain synthetic peptides. It is possible that tyrosine is important in the secondary binding effects in pepsin action. Kitson and Knowles (1971c) using phenyl glyoxal, an arginine specific modifying agent, found only 45% of peptic activity remained towards haemoglobin, and a synthetic substrate. Huang and Tang (1972) using

2,3-butanedione showed that an arginine 12 residues from the C-terminus is modified with a resulting 85% loss in activity. Thus although it cannot be directly involved in the catalytic process, may be important perhaps in assisting an ionisation at the active site.

Lokshina and Orekhovich (1964) have shown that tryptophan and methionine, do not appear to be catalytically important.

1.6 Introduction to Pepsin C.

Pepsin C, originally called parapepsin II, was first isolated from crude porcine pepsin by Ryle and Porter (1959). Porcine pepsin C has been found to be similar in many respects to pepsin A. Its amino acid composition (Ryle and Hamilton 1966) shows many similarities to pepsin although the aspartic acid to glutamic ratio is reversed and pepsin C has four lysines and four arginines compared to one and two respectively in pepsin A. This may partly explain the greater stability of pepsin C at pH 6.9. Pepsin C has no phosphate group. The greatest difference in terms of activity is the inability of pepsin C to catalyse the hydrolysis of acetyl phenylalanyl diiodotyrosine, which is a good substrate for pepsin A.

An equivalent enzyme to pepsin C has been described in some other species, for example, gastricsin, isolated by Tang *et al.* (1959) from human gastric juice, and also enzymes from the smooth dogfish (Merrett *et al.* 1969) and chicken (Bohak 1969). It is believed to arise from the pyloric cells of the stomach. However a pepsin C is apparently absent from the Japanese monkey (Kageyama & Takahashi 1976).

Some studies on the active site of pepsin C (Kay and Ryle 1971) using diazoacetyl norleucine methyl ester as a specific inactivator, showed a single aspartyl residue in the sequence Ile-Val-Asp-Thr was modified. This shows a further similarity with pepsin A (see section 1.5). Ryle (1960b) has demonstrated transpeptidation of the amino transfer type with pepsin C. Different reports have been made concerning the molecular weight of pepsin C. For the porcine enzyme, Ryle and Hamilton (1966) report a molecular weight of 36,000 representing 332 residues, while Chiang et al.(1967) give a value of 32,500 from ultracentrifuge data, and their amino acid analysis suggests a total of 304 residues. According to Mills and Tang (1967), human gastricsin is slightly smaller than the porcine enzyme with a molecular weight of 31,500.

The side chain specificity of pepsin C with reference to the points of cleavage on the B chain of oxidised insulin has been studied by Ryle and Porter (1959). One of the major sites of action is the tyrosyl-leucyl bond at position 16-17, which comes in the sequence -Glu-Ala-Leu-Tyr-Leu-Val-CyS(O_3 H)-. The alanyl-leucyl bond in the same sequence is also quite susceptible. Some preliminary studies (Ryle et al. 1968) showed pepsin C would hydrolyse the peptide Z-Ala-Leu-Tyr-Leu-Val-(Sbenzyl)-CySNH_2, (where all residues are of the L configuration) and Ryle (unpublished) has found hydrolysis at the Tyr-Leu bond in the peptides Ac-Tyr-Leu-Val-His-Methyl ester, Ac-Tyr-Leu-Val-picolylester and Ac-Tyr-Leu-Val-CySO_3H.

The aim of this project has been to prepare a series of related peptides based on the residues 14 to 18 in the sequence of the

insulin B chain, with some alterations to the residues, and to test their suitability as substrates for pepsin C. Some detailed kinetic studies were carried out with suitable peptides to try and gain some further information about the active site of the enzyme.

The peptides were also used to compare the relative activities of the porcine pepsins A and C and their human equivalents. It was hoped that one of the peptides might be a suitable substrate for gastricsin but not for human pepsin, so that an assay system might be developed using this and the pepsin A sensitive peptide Ac-Phe-Tyr(I_2) to measure the relative amounts of each enzyme in a mixture. This may have clinical applications in studying the levels of the two enzymes in the gastric juice of patients with gastric disorders, in a system which may be readily automated. Peptides which have so far been found for gastricsin (Huang & Tang 1969) were only very slowly hydrolysed, although in peptides Z-Tyr-X, where X was alanine, threonine, leucine or serine, the bond was susceptible to attack by gastricsin but not by pepsin. This shows similarity to the resistance of Tyrosyl-X bonds found by Fruton.

SECTION 2. GENERAL METHODS

2.1 Determination of pepsin activity using bovine haemoglobin as substrate

Anson and Mirsky (1932) used haemoglobin as a substrate to measure the activity of proteolytic enzymes. The method has been modified, (Anson 1938; Northrop et al. 1948; Ryle & Porter 1959; Tang 1970).

a) Method of Ryle and Porter (1959)

This method was used for the assay of porcine pepsins. Bovine haemoglobin enzyme substrate powder was purchased from Armour Pharmaceuticals Ltd. 5 g of the powder was dissolved in about 100 ml of distilled water and this solution was dialysed against two lots of distilled water (2 l. each), to remove low molecular weight material, which otherwise causes a high blank value in the assay. The dialysed solution was made up to 200 ml with distilled water, thus giving a 2½% (w/v) solution of haemoglobin. This was filtered through Whatman no. 54 filter paper and stored at 4°C, after adding a little thiomersal as a preservative.

Assays were performed in duplicate at 37°C. Some acid denatured haemoglobin was prepared by incubation, at 37°C, of a mixture of 2½% haemoglobin solution and dilute hydrochloric acid in the ratio 4 : 1 for 15 minutes. The concentration of the acid employed was such that there was a final excess in the incubation mixture of 0.06 millimoles. 1 ml of the denatured haemoglobin was added to 0.2 ml of enzyme solution (either aqueous or buffered

at a concentration of about 0.01 mg ml^{-1}), which had been equilibrated to 37°C. Blank tubes were set up using 0.2 ml water or buffer as appropriate. It is not necessary to perform an enzyme blank since the value obtained is negligible in comparison with that measured for the substrate blank. After 10 minutes exactly, the reaction was stopped by the addition of 5 ml of a 4% (w/v) solution of trichloroacetic acid. The tubes were shaken and then left to stand for 10 - 15 minutes to ensure complete precipitation of insoluble protein fragments. The contents were filtered through Whatman no. 3 filter paper, and the absorbances read at 280 nm in a Unicam SP 500 spectrophotometer. The difference in absorption between the mean 'live' assay and the mean blank values was a measure of the activity of the enzyme sample. This could be converted to milliproteolytic units of haemoglobin digesting activity ($m[PU]^{Hb}$) using a standard curve (Ryle 1960). Pepsin A shows a linear response of ΔE 280 with activity, but that for pepsin C is non linear, and a fairly narrow portion of the curve is used to determine the proteolytic activity. The specific activity is expressed in terms of $m[PU]^{Hb}$ per ml/E280 enzyme solution.

b) Method of Tang (1970)

This method was used for the assay of human pepsins. Volumes were scaled down to avoid waste of materials. A 10% (w/v) solution of haemoglobin was prepared in a similar manner to that described above. The haemoglobin was acid denatured by the addition of 7 ml 0.3 M HCl to 20 ml of the 10% haemoglobin solution. This mixture was made up to 100 ml with water. (The 2½% solution described above could be used by adjusting the relative amounts of haemoglobin

solution and water). The pH of the solution was measured, and if necessary was adjusted to within the range pH 3.0 - 3.2.

0.2 ml enzyme solution in water or dilute buffer and 0.2 ml 0.1 M citrate buffer pH 3.1 were incubated together at 37°C. 2 ml of acid denatured haemoglobin were added and the reaction allowed to proceed at 37°C for 10 minutes. 4 ml of a 5% (w/v) solution of trichloroacetic acid was then added to stop the reaction, and the assay continued as described above. Duplicates of each sample were performed and also a duplicate blank, using water in place of enzyme. The activity is expressed as Δ E280/ml representing the observed difference in absorbance between the live and blank assays for one millilitre of undiluted enzyme solution. The specific activity may be expressed as $\frac{\Delta \text{ E280/ml}}{\text{E280 of enzyme solution}}$.

2.2 Determination of pepsin activity using acetyl phenylalanyl-diiodotyrosine (Ac-Phe-(I_2)Tyr) as substrate.

a) Method of Ryle and Porter (1959)

Duplicate 0.5 ml portions of enzyme solution containing a maximum of 50 µg pepsin, and 0.25 ml hydrochloric acid were incubated for 10 minutes at 37°C. The concentration of the hydrochloric acid was such to give a final overall concentration of 0.01 M acid in the incubation mixture (i.e. pH 2.0). 0.25 ml of a solution of 1.0 mM Ac-Phe-(I_2)Tyr in 0.01 M. sodium hydroxide was added and the reaction allowed to proceed for 20 minutes. The reaction was stopped by the addition of 1 ml of ninhydrin reagent (the modified reagent of Moore and Stein 1954), and the solution heated at 100°C in a water bath for 15 minutes. After cooling 5 ml of 60% (v/v) aqueous

ethanol was added and the absorbance read at 570 nm in a Unicam SP500 spectrophotometer. Blanks were performed in which ninhydrin reagent was added to stop the action of the enzyme before the addition of substrate. Activities are expressed in ΔE570/ml enzyme, or µmoles diiodotyrosine produced/minute/ml enzyme. The ninhydrin reagent was kept in an amber glass dispensing unit, under nitrogen.

b) Method of Tang

The incubating conditions of Tang (1970) were used for the human enzymes, the method being adapted to allow use of the same stock solution of APD as used above, and also using the ninhydrin reagent described above, since no difference was observed between the Rosen reagent used by Tang and the Moore and Stein reagent.

50 µl enzyme solution in water or buffer (10 - 50 µg pepsin) were incubated with 0.45 ml of 0.0239 M hydrochloric acid at 37^{o}C. 0.5 ml APD solution was added and the reaction allowed to proceed for 1 hour. The final pH of the incubation mixture is calculated to be 2.24. The reaction was terminated, and the assay continued as above. This method does not use different acid concentrations to counteract any buffer in the enzyme solution, for example the citrate buffer used in the enzyme preparation, but was nevertheless used for the human enzymes. This method was mostly applied to the assay of aqueous enzyme solutions in the attempt to set up a differential assay for the human enzymes.

2.3 Preparation of gel filtration and ion exchange media

Sephadex G100 (Pharmacia Ltd) Amberlite CG50 (BDH) and DEAE cellulose (Whatman) were the media employed.

A suitable quantity of the appropriate material was poured as a slurry into the column, and allowed to settle. The resins were equilibrated with the appropriate buffer, and fractions of constant volume were collected by a Central fraction collector. Regeneration of the ion exchange resins was by washing with NaOH (1.0 M for the Amberlite and 0.1 M for the DEAE cellulose) and then washing with water to neutrality. The Amberlite resin was washed with 1.0 M HCl and again with water before equilibrating with buffer. DEAE cellulose was directly equilibrated with the appropriate buffer.

2.4 High voltage paper electrophoresis

Spots or streaks of the material to be investigated were applied to origins on Whatman no. 1 or Whatman 3 MM chromatography paper as appropriate. The papers were run in buffers at

pH 2.0 (0.75 M formic acid and 1.0 M acetic acid respectively)

pH 3.6 (pyridine 10 ml, acetic acid 100 ml made up to 2l. with water)

pH 6.5 (pyridine 100 ml, acetic acid 4 ml made up to 2l. with water)

in either immersion tanks using white spirit as coolant, or in a Miles High-Volt flat bed electrophoresis machine. The papers were dried in an oven at about 60°C and were sprayed first with ninhydrin solution (0.25% w/v in ethanol), which after heating for a few minutes at 100°C located compounds with free amino functions, and then with Pauly reagent to locate histidine and tyrosine containing peptides. Pauly reagent was prepared by mixing equal volumes of a

1% (w/v) solution of sulphanilic acid in 1.0 M HCl and 5% (w/v) sodium nitrite and allowing the mixture to diazotise at room temperature for a few minutes. The mixture was sprayed onto the electrophoresis paper and the colour developed by spraying with a 5% (w/v) solution of sodium carbonate. Occasionally, detection of materials was attempted using a spray of sodium hypochlorite containing 1% available chlorine. After drying the paper it was sprayed with a solution of starch/potassium iodide (equal proportions of 1% w/v solutions). Grey-black spots corresponding to material on the electrophoretogram developed, but the method was not very reproducible or reliable.

2.5 Polyacrylamide gel electrophoresis

This was performed using a Shandon gel electrophoresis kit. Gels were prepared in glass tubes 0.5 x 7.5 cm which had been washed thoroughly with chromic acid and water. The tubes were closed at one end with parafilm and filled to within 1 cm of the top with a solution made as follows:- 13.4% (w/v) acrylamide and 0.48% (w/v) methylene bis acrylamide in water, 0.5 volumes; distilled water, 0.3 volumes; 0.1 M acetate buffer pH 5.6, 0.2 volumes. These solutions were mixed and degassed and then 0.01 volumes each of 10% (w/v) TEMED and ammonium persulphate were added to initiate the polymerisation.

The liquid in the tubes was carefully overlaid with a little degassed water to make a level surface to the gel, and to prevent oxygen contact, which inhibits the polymerisation process. When set, the parafilm was removed from the tubes, which were then installed in the apparatus. The upper and lower reservoirs were

filled with 0.1 M acetate buffer pH 5.6, ensuring no air bubbles became trapped in the tubes.

20 - 50 µg protein was applied to each gel in up to 100 µl of 10% (w/v) sucrose solution, which also contained 5 µl bromophenol blue (0.1 w/v) to act as a marker. The electrophoreses were carried out at approximately 80 volts and 5 milliamps per tube for about 1 hour.

Staining procedures for:-

a) protein

The gels were removed from the tubes and fixed with 12½% (w/v) trichloroacetic acid for 2 hours. The gels were placed in 0.1% Coomassie brilliant blue (R) in 12½% trichloroacetic acid for 2 hours and then transferred to 12½% trichloroacetic acid to remove stain from regions of the gel where there was no protein.

b) activity

A modification of the method of Uriel (1960) was used. After being removed from the tubes, the gels were incubated at 37°C for 15 minutes in acidified haemoglobin solution, (4 : 1 mixture of 2½% haemoglobin solution (section 2.1) and 0.39 M HCl), followed by 15 minutes incubation in 0.01 M HCl. This allows absorbed haemoglobin to be digested in the region of the enzymes. The haemoglobin remaining in the gel was fixed in 12½% (w/v) trichloroacetic acid for half an hour and then stained by transferring the gel to a 0.1% (w/v) solution of amido black in 1.0 M HCl for 10 minutes. Destaining of the region where digestion had occurred was by 1.0 M acetic acid.

Positions of the protein and enzyme containing regions were measured from the top of the gel, and the gels were also scanned in an SP 800 spectrophotometer.

2.6 Amino acid analyses

0.5 ml of solution containing about 250 nmol peptide, and 0.5 ml 12 M HCl were frozen and then thawed in a test tube to remove air from the solution. The solution was frozen again, and the tube sealed under vacuum. Hydrolysis was allowed to take place at 105°C for 24 hours. The tubes were allowed to cool, opened and the contents were dried in a desiccator, and taken up in pH 2.2 buffer for application to the amino acid analyser. Either a Locarte, LKB or Beckmann analyser was used. I am grateful to Mr. Auffret for assistance with some of the analyses.

2.7 Purification of porcine pepsins A and C

The enzymes were prepared by Dr. Ryle and Mr. J. Cockrill. Three preparations of pepsinogen C (method of Ryle 1960a) were pooled, and activated to pepsin C at pH 2.0. Activation was stopped by increasing the pH to pH 4.4 and pepsin C was separated from the activation peptides by chromatography on S-E Sephadex C25.

Pepsin A was obtained as a by product from a preparation of pepsin D from crude pepsin (Hopkins and Williams, pepsin B.P.). The pepsin was then chromatographed on Sephadex G.100.

SECTION 3. METHODS USED IN PEPTIDE SYNTHESES

3.1 Purification of dimethyl formamide

In order to ensure that the solvent, used extensively in the peptide syntheses, was free from dimethylamine and formic acid, a regular purification procedure was adopted. Dimethylformamide was stirred overnight with potassium carbonate and was then refluxed over phthalic acid in a nitrogen atmosphere under reduced pressure for about 30 minutes. The solvent was then distilled under nitrogen using a fractionating column at about 45°C and at 15 mm of mercury pressure. The first 5% of the distillate was discarded. A test for the absence of amines was done by the method of Stewart and Young (1969). Equal volumes of a 1 mg per ml solution of fluoro-dinitrobenzene in ethanol and dimethyl formamide were mixed and allowed to stand for 30 minutes. The absorbance of the solution was then read at 381 nm. The dimethyl formamide should have a value of no more than 0.1 or 0.15 units above an FDNB blank. The solvent was found to keep satisfactorily for a few weeks after distillation.

3.2 Preparation of ion exchange media

a) Amberlyst 15

This macroreticular cation exchange resin was purchased from BDH Ltd. and has a capacity of 1.75 milliequivalents per ml. Regeneration was by washing with M NaOH, distilled water, M H_2SO_4, and water, and the resin was stored as the acid form under methanol. Prior to the use of the resin a column was poured and washed with

several volumes of ethyl acetate and was then equilibrated with a solution of 3-bromopyridine or pyridine in ethyl acetate, as described in a later section.

b) Amberlite IR4B

A slurry of the resin was poured into a column and it was used in the acetate form. The resin was regenerated by washing with 1.0 M HCl, water, 1.0 M NaOH, water, 1.0 M acetic acid and finally glacial acetic acid.

3.3 Thin layer chromatography

5 x 20 cm plates were prepared using Kieselgel G and Kieselgel HF 254 (9 : 1), about 1 gm of dry silica in 2.5 ml water being used per 100 cm^2 of plate. The solvent systems used are given below. After developing the chromatogram and drying, the plates were "stained" with ninhydrin and Pauly reagent (as described for paper electrophoresis, section 2.4). The plates were then placed in an atmosphere of chlorine for 5 minutes causing bleaching of the plate and reaction of the chlorine with many chemical groups. After allowing absorbed free chlorine to be removed from the plate, they were sprayed with a starch/KI solution (1 : 1 mixture of 1% w/v solutions) yielding grey/black spots.

The compositions of the chromatography systems used are shown below (G.T. Young, personal communication).

E_3	Methanol, chloroform	1 : 3 (v/v)
E_4	Methanol, chloroform	1 : 9 (v/v)

G_3 Pyridine, acetic acid, water (20 : 6 : 11) by volume

and

Ethyl acetate 1 : 3 v/v

Elemental analyses were performed at the Microanalytical Laboratory, Oxford.

SECTION 4 PEPTIDE SYNTHESIS

4.1 Introduction to the methods used

Peptide bonds were formed between the α amino group of a carboxyl protected amino acid or peptide, and the activated carboxyl group of an amino protected amino acid. The coupling was achieved in solution, using dicyclohexyl carbodiimide, introduced into peptide chemistry by Sheehan and Hess (1955), with the addition of 1-hydroxybenzotriazole. König and Keiger (1970) reported that this reagent and some derivatives of it, promoted the coupling, probably by the formation of an active ester, and also helped to prevent racemisation and the formation of N-acyl ureas which are unwanted side products. Khorana (1955) had reported that the dicyclohexyl carbodiimide method resulted in the production of those side products with only a low yield of the desired peptide.

Amino group protection was by a t-butyloxycarbonyl (Boc) blocking group, proposed by McKay and Albertson (1957) and Anderson and McGregor (1957) as an alternative to the benzyloxycarboxyl (Z) group which had been widely used to that time. The t-butyloxycarbonyl group provided some simpler routes in the synthesis, being readily cleaved in acid conditions. Synthesis of the protected amino acids was with t-butylazido formate (Carpino 1957) using the pH stat method of Schnabel (1967).

Carboxyl protection was by an ester or an amide group.

The main advantage of using a homogeneous phase for the coupling stage is that completion of the reaction can be checked

at each stage, which is not possible when the peptide is covalently linked to a polymeric support as in the 'solid phase' method of Merrifield (1963). However, one important advantage of the solid phase method is that removal of coproducts and excess reagents is simply by washing the resin, to which only the peptide is attached. These two important features of the homogeneous and solid phase methods of synthesis can be combined if the peptide is reversibly attached to an insoluble supporting medium. This principle was introduced by Camble et al. (1968) who used the basic picolyl ester group of the peptide concerned as a means of reversibly attaching the product molecules formed in homogeneous phase to a cation exchange resin, where they were purified. Sulphoethyl Sephadex was originally used, but this was found to cleave the amino protecting group. The use of Amberlyst 15 resin was introduced by Burton and Young (1971) in non aqueous media. The use of the basic picolyl ester group as a means of attaching the product to the Amberlyst resin was extended by Schafer and Carlsson (1972) to include the use of a histidine residue in the peptide as the basic group. Since cationic histidine peptides have been used successfully by Fruton (section 1.4) and others in investigating pepsin action, it was decided to use a histidine residue at the C terminus of all the synthetic peptides.

4.2 General scheme for synthesis

Some technical notes for peptide synthesis were kindly provided by Dr. G. T. Young and these were used as a guide. Purification procedures for solvents, ion exchange media and the thin layer chromatography techniques and solvent systems are described in

sections 3.1 - 3.3.

L-amino acids were used throughout the syntheses.

a) Deprotection of the amino terminus

The peptide was dissolved in a small volume of anhydrous trifluoroacetic acid and the solution was allowed to stand at room temperature for 10 - 15 minutes. Thin layer chromatography in system G_3 at this stage shows a new fast developing ninhydrin positive spot and disappearance of the original Boc-peptide. However, the trifluoroacetyl salt caused rather a streak in the system used, and at a later stage, after evaporation of excess acid at 0.5 mm Hg pressure and trituration with dry ether to remove any remaining acid, chromatography of the resulting solid, sometimes showed a little remaining Boc-peptide. If this occurred the deprotection stage was repeated. A small loss of peptide occurred during the trituration stage, some apparently dissolving in the trifluoroacetic acid/ether mixture. The lost peptide could be partly recovered by evaporation and retrituration.

b) Activation of the carboxyterminus

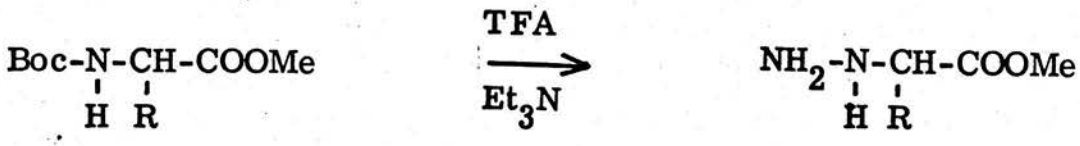
The Boc amino acid was dissolved in a few millilitres of dimethyl formamide. A 1.2 - 1.5 M excess of N-N dicyclohexylcarbodiimide and hydroxybenzotriazole were added. The reaction was allowed to proceed at room temperature for about 30 minutes.

c) Coupling

The trifluoroacetate salt of the deprotected peptide was dissolved in dimethyl formamide and two equivalents of triethylamine

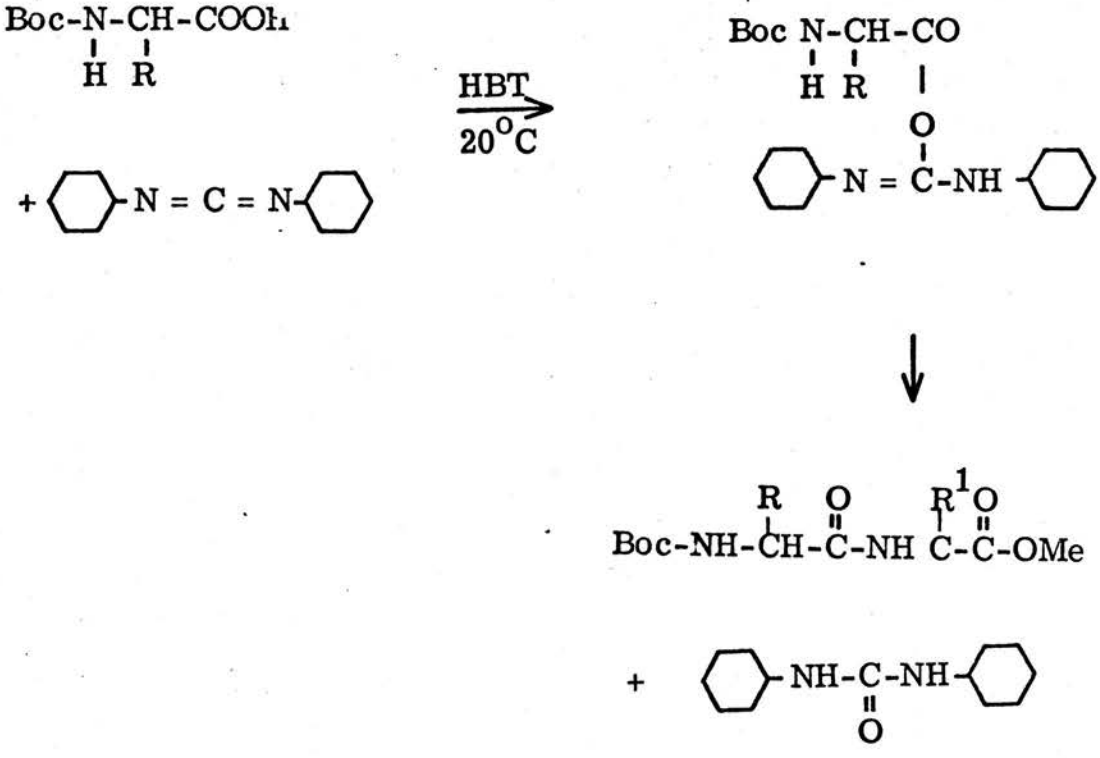
FIGURE 4 Typical reaction scheme

1) Deprotection of N-terminus



+

2) Activation of C-terminus



were added to release the N-terminus. Excess triethylamine was evaporated until damp indicator paper no longer showed a basic atmosphere just above the surface of the solution. The activation mixture was added and a small sample was taken after about 5 minutes to test for product by thin layer chromatography. Further samples were taken later for testing the completeness of the reaction, and if necessary after about five hours, some more activated carboxyl group was added. The reaction time depended partly on the residue being added, Boc-phenylalanine and Boc-tyrosine for example reacting more slowly than Boc-alanine.

The dimethyl formamide used as solvent was difficult to remove thoroughly by evaporation before running the thin layer plates, and sometimes this led to bad running of the spots, which could give rise to uncertainty of the Rf values. The chemical reactions of a typical synthesis are represented in figure 4, and Table 2 lists the typical Rf values of the various components of peptide synthesis in thin layer chromatography. Values varied slightly, probably due to slight differences in the thickness of the silica, the temperature at which the plates were developed, and the absolute composition of the solvent system, which was not made up freshly for every chromatograph.

d) Separation of product

After the coupling reaction was apparently complete, the dimethyl formamide was partially evaporated, to a volume of about 10 mls. 15 - 20 ml of ethyl acetate was added and the mixture allowed to cool at $4^{\circ}C$ for about one hour, to allow maximum precipitation of the dicyclohexyl urea, which was filtered off.

TABLE 2

Typical Rf values of reagents and intermediates

in peptide synthesis

Compound	Detection	Rf in system G_3	Rf in system E_4
Boc-amino acid	ninhydrin (weak) Cl_2	0.75-0.8	streak
Boc-amino acid OBt*	ultra-violet ninhydrin (weak) Cl_2	0.85	0.8
Hydroxybenzo-triazole	ultra-violet Cl_2	0.6	streak at origin
Dicyclohexyl urea	ninhydrin	0.9	0.85
3-Bromo pyridine	ultra-violet	-	0.65
Pyridine	ultra-violet	-	0.5
NH_2-peptide-ester or amide	ninhydrin (strong) Pauly Cl_2	streak at origin	
Boc-peptide (product)	ninhydrin (weak) Pauly Cl_2	0.3 0.5	0.2-0.4

The composition of systems G_3 and E_4 are given in section 3.3.

*OBt - hydroxybenzotriazole ester.

Due to the slight solubility of dicyclohexyl urea in this system the theoretical weight of material was not collected at this stage.

In general, the filtrate was applied directly to a column of Amberlyst 15 resin in its 3-bromopyridinium or pyridinium form, and allowed to pass through slowly. If product was detectable in the effluent this was either reapplied to the column, or passed through a second smaller column, until all the product was retained. The resin was then washed thoroughly with ethyl acetate (7 - 8 column volumes), dimethyl formamide, to remove side products and the product was then eluted with 13% v/v collidine/dimethyl formamide mixture. A final elution with triethylamine was sometimes used after no more product was removed with collidine. Product was sometimes found in the eluate but this was often accompanied by more basic material which showed positive ninhydrin reaction in thin layer chromatography, but was not further identified. The solvents were evaporated at reduced pressure (0.5 mm mercury pressure) and product was obtained as a solid after trituration with ether to remove the last traces of solvent.

Some problems were encountered in the use of the Amberlyst resin. The new resin was initially washed in methanol and it released a brown colour into the solvent. Washing was continued until no more colour appeared, and the resin was put through a regeneration cycle as described in section 3.2a, before use. However the first pilot preparations of peptides seemed to become rather contaminated with the brown material. Eventually the resin became free from the contaminant, for which the manufacturers offered no explanation, while still functioning as required for the purification procedure.

During the application of the product solution and washing of the column, a little product usually appeared to become associated with the hydroxybenzotriazole and did not attach to the column.

In the initial preparations, the reaction mixture was taken down to dryness and then redissolved in ethyl acetate. Extraction with aqueous sodium bicarbonate was tried, in an attempt to remove the hydroxybenzotriazole. The ethyl acetate phase was washed with water, dried and then applied to the column. Deesterification of the peptide seemed to occur during contact with the sodium bicarbonate extraction and was subsequently omitted. The small loss of product lost at the column purification stage was therefore tolerated. The large peptides were not soluble in ethyl acetate and so the bicarbonate wash could not, in any case, be used.

The yields of peptides were low to begin with, probably mainly due to unfamiliarity with the techniques. The yields improved with experience but were still lower than has been reported for the method by Dr. Young and coworkers.

4.3 Laboratory syntheses

a) t-butyloxycarbonyl azide (Boc-azide)

This was prepared by the method of Carpino et al. (1959). 100 gm t-butyl carbazate, 80 gm glacial acetic acid and 125 ml distilled water were cooled in an ice bath with stirring. 57.4 gm sodium nitrite dissolved in 80 ml water were added over an hour, keeping the temperature in the range 9 - 13°C. Stirring was continued for 30 minutes and 125 ml of water were added. The azide layer was separated and the aqueous layer was extracted with

four 40 ml portions of ether. The combined organic layers were washed with water and 1 M sodium bicarbonate solution, and dried over magnesium sulphate. The solvent was removed at 40°- 45°C 140 mm mercury pressure and then at 22°C 20 mm mercury pressure. The yield was 84.3%.

b) Boc-amino acids

The pH stat method of Schnabel (1967) was used. 0.05 moles of amino acid were suspended in 20 ml of 50% (v.v) dioxan/water. Peroxides were removed from the dioxan by passing it through a column of alumina typeHand their absence was shown by saturated potassium iodide solution remaining colourless when mixed with the dioxan. 0.055 moles boc-azide were added in several portions. The reaction was allowed to proceed until no more of the 4 M sodium hydroxide from the burette was being taken up. The setting of the pH stat was according to the amino acid derivative being prepared. It was necessary to flush the reaction mixture with nitrogen to prevent carbon dioxide being taken up from the atmosphere.

When the reaction was apparently complete, the reaction mixture was extracted with ether to remove unreacted azide. The aqueous phase was chilled, acidified to pH 3 with citric acid and extracted with ethyl acetate. This was then washed with water, dried over magnesium sulphate, and the solvent evaporated. Partition towards ethyl acetate from the citric acid was not very favourable and prolonged extractions were required.

Although the reactions appeared complete, by cessation of base uptake, after extraction very low yields of product were found, the unreacted amino acid remaining in the acid phase. Provided

sufficient product was obtained for the anticipated peptide preparations the low yields were tolerated. It is not clear why the reactions did not go to completion, perhaps even longer times were required. The boc-azide may have still contained some solvent, although adding more of the reagent did not seem to cause any further reaction. Distillation of the boc-azide was not recommended due to the risk of explosion.

(i) t-Boc-L-alanine

The reaction was carried out overnight at pH 9.8

Yield 30%. Melting point 80°-81°C (lit 82-3°).

Chromatography in system G_3. Rf = 0.78.

(ii) t-Boc-L-leucine

The reaction took several days at pH 10, adding boc-azide in batches over the period. Evaporation of the ethyl acetate phase yielded a brown oil, which after trituration with 40°-60° petroleum spirit, yielded boc-leucine.

Yield 67%. Melting point 85°-86°C (lit 86°-7°).

Thin layer chromatography - homogeneous in G_3. Rf = 0.75.

(iii) t-Boc-(O-benzyl)-L-tyrosine

25 mmoles O-benzyl-tyrosine (see below) was suspended in the aqueous dioxan, but would not react very well with the boc-azide. The benzyl tyrosine remained in suspension and was filtered off before the extraction procedure. (17 mmoles recovered). 5.8 mmoles of required product were finally isolated.

Yield 23% . Chromatography: G_3. Rf = 0.81.

c) 1-Hydroxybenzotriazole

0.3 mole O-chloronitrobenzene, 1 mole hydrazine hydrate and 167 ml ethanol were refluxed together. 670 ml of water were added

and unused reactants extracted with ether. The aqueous phase was acidified with concentrated hydrochloric acid, with vigorous stirring. The crystals were collected and recrystallised from ethanol.

Yield 58% (Monohydrate). Melting point 156°C (lit 157°C)

Chromatography: G_3. Rf = 0.54.

d) O-(Bzl)-L-Tyrosine

8 mmoles tyrosine were dissolved in 67.5 ml, 2.0 M sodium hydroxide and 4 mmoles $CuSO_4 . 5H_2O$ in aqueous solution were added. Since the sodium hydroxide necessary to dissolve the tyrosine was 27.5 ml in excess of that described by Wunsch et al. (1958); sufficient sulphuric acid was added to neutralise this excess. The copper complex was collected and dried. Yield 82% . The complex was dissolved in a mixture of 127 ml of water, 416 ml methanol and 22 ml 0.3 M NaOH. 7.84 ml benzyl bromide was added, and the mixture was shaken vigorously for about one hour. O-benzyl tyrosine was precipitated and collected. Yield 67%. The product was washed with methanol/water (1 : 3.5 v/v) and recrystallisation from 80% acetic acid.

Yield 32%. Melting point 218°-221°C (lit 222°C).

Chromatography: G_3. Rf = 0.12.

e) Acetyl-L-Phenylalanine

30 mmoles L-phenylalanine were suspended in water. The pH of the solution was brought to pH 8.0 with 2.0 M sodium hydroxide. 0.2 ml portions of acetic anhydride were added to the suspension with stirring, the pH being maintained at pH 8.0 using a pH stat. Acetic anhydride was added until the rate of base uptake was

apparently due solely to hydrolysis of the anhydride itself, this having been measured in a previous experiment. A total of 33.8 mmoles of acetic anhydride were used and double this amount of base. The solution was filtered and acidified with 5.7 M HCl and cooled. The precipitated acetyl phenylalanine was filtered and washed with ice cold water.

Yield 90%. Melting point 168° - 169°C (lit 169 - 170°)

$[\alpha]_D = 45.5°$ (c = 2% EtOH) (lit 46.1°, Kitson & Knowles, 1971a) 44.5°, Ryle, unpublished).

Chromatography: G_3. Rf = 0.45. E_4. Rf = 0.63.

% composition calculated C 63.8%, H 6.38%, N 6.76%.

Found C 63.9%, H 6.53%, N 6.65%.

4.4 Synthesis of the peptides

The general procedure for the syntheses has been described (section 4.2). The peptides which have been prepared are shown below, some specific details of each preparation being given. Amino acids of the -L- configuration have been used throughout.

a) Boc-Ala-Leu-Val-His-methyl ester

The synthesis of the peptide followed the general methods previously described, starting with 3.0 mmoles of histidine methyl ester dihydrochloride. Two equivalents of triethylamine in dimethyl formamide were added to the amino acid to generate the free base. The solution obtained was added to the activation mixture containing 4.5 mmoles of boc-valine. The coupling was complete after 30 minutes.

A sodium bicarbonate wash was used for this peptide to try to remove excess of hydroxybenzotriazole from the ethyl acetate solution.

Some peptide also seemed to be removed from the ethyl acetate phase, and this was partly recovered by re-extracting the alkaline solution with ethyl acetate. The 3-bromopyridinium form of the column was used, and finally yielded an oily substance. The product was partly precipitated with 40-60° petroleum spirit. Thin layer chromatography in system G_3 (section 3.3) showed product with Rf of 0.45 and in E_4 , Rf = 0.31. The material was used without further purification in the next step of the synthesis. The peptide boc-Leu-Val-His-methyl ester was prepared according to the general method, again using a sodium bicarbonate wash. The product Rf in system G_3 of 0.51 and E_4 of 0.35. Yield at this stage was 27%.

After the next coupling stage, the sodium bicarbonate wash was omitted and the peptide boc-Ala-Leu-Val-His ester obtained. Rf in system G_3: 0.48, E_4, 0.20.

It was found that this type of peptide would be unsuitable for kinetic studies since release of the boc-group occurred on boiling with ninhydrin reagent (see experimental section for assays used) and a high blank value was obtained. Hence the peptide was converted to the N-acetyl form.

The amino group was released by the addition of a solution consisting of 3 ml 45% (w/v) hydrogen bromide in glacial acetic acid, and 5 ml 0.04 M phenol in glacial acetic acid. The phenol was added to remove any free bromine from the solution. The solution was allowed to react for 1 hour after which excess of reagent was evaporated away. The residue was dissolved in dimethylformamide and triethylamine was added to release the free base. One equivalent of acetic anhydride was added and the solution stirred for 1 hour.

Unfortunately two spots were found to be present upon thin layer chromatography E_4, 0.075, 0.27. The relative mobilities of the two components on paper electrophresis at pH 6.5 suggested that partial deesterification had taken place. Treatment of a small portion of the peptide with hydroxybenzotriazole and dicyclohexyl carbodiimide in methanol resulted in a single spot on thin layer chromatography with an Rf value corresponding to that of the ester. However, this method could not be used for reesterification of the whole batch because of the risk of racemization of the C-terminal residue. Preparative thin layer chromatography was carried out in system G_3, and finally yielded 40 mg peptide, (3% based on histidine methyl ester dihydrochloride).

b) Large scale preparation of Boc-Leu-Val-His-methyl ester

The peptide was prepared commencing with 10 mmoles of histidine methyl ester dihydrochloride. The coupling with activated boc-valine took about 2 hours. Separation of the product followed the method described in section 4.2d, the filtrate (50 ml) being applied to a 2.5 x 30 cm column of Amberlyst 15 which had been equilibrated with a 10% (v/v) solution of 3-bromopyridine in ethyl acetate. The final eluate containing the product after the normal washing and elution procedures was left standing for some days, and on evaporation yielded a brown oily residue. This was taken up in methanol, and activated charcoal was added to decolourise the solution. This may have resulted in some loss of product.

Chromatography in system G_3 showed product Rf 0.5 and a little hydroxybenzotriazole. After evaporation of the methanol it was decided to proceed directly to the next stage.

Deprotection of the boc peptide, and coupling with activated boc-leucine was carried out as described in the general methods section, the coupling taking several hours for completion. It was found during the column purification of the peptide that not all the peptide exchanged with 3-bromopyridine, and so the effluent was applied to a second column (2 x 18 cm) to try and recover the product. The product was finally obtained as a buff-coloured solid.

Yield 64%, based on histidine methyl ester, dihydrochloride.

Chromatography in system G_3, Rf 0.55; E_4, 0.35.

This peptide was used as the starting point for all subsequent preparations.

c) Ac-Tyr-Leu-Val-His- methyl ester. I

(i) Boc-(Bzl)Tyr-Leu-Val-His-methyl ester

0.64 mmoles of the tripeptide ester were deprotected and coupled with boc-(OBzl)tyrosine. Overnight treatment was required for complete reaction. The Amberlyst 15 resin was equilibrated with pyridine in ethyl acetate (Schafer & Carlsson 1972) for this, and all subsequent preparations.

Chromatography in G_3: Rf = 0.5 - 0.6 (streak); E_4, Rf = 0.39.

(ii) Acetylation

Deprotection of the boc-peptide was carried out by solution in anhydrous trifluoroacetic acid for 15 minutes. Hydrogen bromide in glacial acetic acid was not used, to avoid the risk of bromination of the tyrosine nucleus. Deprotection with trifluoroacetic acid was adopted for all subsequent acetylation steps, as it was found to be more generally convenient, despite the need for an additional ion exchange step. Excess acid was evaporated at reduced pressure, with final traces being removed by trituration with dry ether. The

off-white solid produced was dissolved in 5 ml glacial acetic acid and passed through a column (10 x 1.5 cm) of Amberlite 1R4B resin in the acetate form to exchange the trifluoroacetate ions associated with the peptide for acetate. This was to prevent trifluoracetylation of the molecule at the next stage. The glacial acetic acid was evaporated away, final traces being removed by trituration with ether, and the residue was dissolved in dimethylformamide. Two equivalents of triethylamine were added, to generate the free base, followed by one equivalent of acetic anhydride. A further equivalent of triethylamine was added over about five minutes to neutralise the acid formed in the reaction. The solvent was removed by evaporation and the product was obtained by trituration with ether.

Yield 78%, based on the tripeptide.

Chromatography: G_3, Rf = 0.28; E_4, Rf = 0.7.

(iii) Debenzylation

The peptide was dissolved in 80% (v/v) acetic acid and about 50 mg 10% palladium charcoal was added. After evacuating the flask, to remove all oxygen from the system, hydrogen was admitted, and the reduction allowed to take place for 2 hours. Excess hydrogen was taken up over the theoretical, perhaps due to a small leak in the system, or to uptake by the catalyst itself. All the peptide was found to be converted after this time, and taking the usual precautions, the solution was filtered and evaporated. The product was crystallised from methanolic hydrochloric acid and ether.

Yield 63%, based on the tripeptide.

Chromatography: G_3, Rf = 0.22; E_4, Rf = 0 - 0.1.

After some preliminary studies it was evident that considerable deesterification had taken place since completing the synthesis. Several solvent systems were tested for use in preparative thin

layer chromatography, in order to separate the ester and free acid from each other, and from the origin. Electrophoresis on thin layer silica plates was also tested but abnormal 'fronts' were seen on the plates and no effective separation of the peptides was observed. The solvent system finally used was G_3 (section 3.3) also including one volume of methanol, which moved the acid from the origin. Although a more volatile system would have been better, none was found.

170 mg of the peptide was applied to two 20 x 20 cm chromatography plates, which were run for one hour. After drying, 1 cm strips at each side of the plate were sectioned off and, covering the rest of the plate with glass, the positions of the ester and acid of the peptides were located by spraying with Pauly reagent. The silica was scraped from the plate in four batches, representing the ester, acid, the region in between these, and the silica between the origin and the acid. The heavy loading of the plates apparently caused poorer separation than previously, and rerunning of the 'in between' fraction which contained both acid and ester was necessary. Elution from the silica was with methanol.

110 mg of free acid and 50 mg of ester were recovered.

(iv) Preparation II

0.8 mmoles boc-Leu-Val-His methyl ester were used for the synthesis which followed the procedure indicated for the first preparation. Coupling was allowed to proceed overnight, and the hydrogenation step took 24 hours.

Yield 90%. Chromatography at each stage showed boc(Bzl)-Tyr-

Leu-Val-His-methyl ester.

G_3: Rf = 0.7, the product associated with hydroxybenzotriazole before column purification.

ac(Bzl)Tyr-Leu-Val-His-methyl ester, E_4: Rf = 0.55

ac-Tyr-Leu-Val-His-methyl ester, E_4: Rf = 0.19.

G_3: Rf = 0.26. Melting point of final product 138°-141°C.

% Composition, calculated for ac-Tyr-Leu-Val-His-methyl ester. acetate. H_2O.

C, 56%; H, 6.94%; N, 12.68%; Found, C, 55.22%; H, 6.82%; N, 12.32%. The slightly low values may be due to a little celite which was used as a filter aid, after the hydrogenation process.

d) Boc-Leu-Val-His amide I

Amides of some peptides were prepared as it was considered that these might be more stable than the corresponding esters.

Ammonia gas was dried by passing it over sodium hydroxide pellets, and it was then bubbled through dried methanol at 0°C for 2 hours. 0.208 mmoles of boc-Leu-Val-His-methyl ester were added to 30 ml of methanolic ammonia. The flask was securely stoppered, and the reaction was allowed to take place at room temperature for 40 hours. After evaporation of solvent, the residue was triturated with 60°-80° petroleum spirit.

Yield 97%. Chromatography: E_4, Rf = 0.195.

Preparation II

0.17 mmoles of the ester was converted to amide.

Yield 97%. Chromatography: E_4, Rf = 0.23; G_3, Rf = 0.4.

e) Ac-Tyr-Leu-Val-His-amide I

0.68 mmoles of the tripeptide were used in the synthesis. After

overnight treatment with activated boc-(Bzl)-tyrosine (BDH) the coupling was apparently complete and the peptide was acetylated as described for the ester, (section 4.4c). The product showed a double spot which was linked with streaking in system G_3, Rf = 0.2 - 0.45. Small scale retreatment with trifluoroacetic acid showed a ninhydrin positive spot at the origin indicating that not all the peptide had been deprotected. After electrophoresis on paper, two product spots which showed similar charges to each other at pH 2, 3.6 and 6.5 were detected. One was thought to possibly represent debenzylated material, a test on the boc(Bzl)-tyrosine used for this preparation having shown two spots. The yield at this stage was 60% from the tripeptide. After hydrogenation for 20 hours in 80% acetic acid, three Pauly positive spots were observed on electrophoresis.

Attempts were made to separate the components using preparative thin layer chromatography in system E_3 (methanol : chloroform 1 : 1). However a mixture of products was found in each fraction collected. It was therefore decided to try an electrophoretic separation. The partially purified fractions were applied to origins on Whatman 3 MM chromatography paper at a loading of 2 mg cm^{-1}. The paper had been washed with 0.1 M acetic acid to remove free amino acids and other compounds which are often associated with the paper. The papers were run at pH 2, on a Miles Hivolt flat bed electrophoresis machine for 360 kilo volt minutes. Staining of marker strips with Pauly reagent showed that loading had been too heavy, and the papers were sectioned and eluted in several fractions in 0.1 M formic acid. Some fractions were rerun and eventually four fractions were obtained, samples of which were hydrolysed and

subjected to amino acid analyses. The major product spot showed the four amino acids in equimolar proportions, and this was the required product. The spot corresponding to the impurity which was originally observed after acetylation, was found not to contain tyrosine. Thus coupling had in fact not gone to completion, although this was not apparent at the time. The two minor components were not characterised fully, still being slightly impure but both apparently contained each of the four aminoacids.

The final yield of required product was unfortunately very low, but some kinetic studies showed that a second preparation would be useful.

Preparation II

The coupling on a 0.4 millimole scale went to completion after the addition of an extra 10% of activated boc-(Bzl)-tyrosine. After the normal column purification procedure and no more product was eluted in the collidine-dimethyl formamide solution, a 10% (v/v) solution of triethylamine in dimethyl formamide was applied. A mixture of components was eluted, including some product. The contaminants were not further identified or removed and the loss of product was tolerated at this stage.

Yield Boc-(Bzl)-Tyr-Leu-Val-His-amide 75%

Chromatography: G_3, Rf = 0.41.

Acetylation was carried out as usual.

Chromatography: G_3, Rf = 0.3 - 0.35 and after hydrogenation 170 mg product was obtained.

Yield 66% (from the tripeptide). Chromatography: G_3, Rf = 0.1, E_4, Rf = 0.023. Melting point: 159°-161°C.

% composition, calculated for Ac-Tyr-Leu-Val-His-amide, acetate H_2O. C, 55.5%; H, 6.95%; N, 15.1%. Found C, 55.87%; H, 7.01%; N, 14.94%

f) Boc-Phe-Leu-Val-His-amide I

0.7 mmoles of the tripeptide were used and for completion of the coupling reaction overnight treatment with a further 20% excess of the activated boc-phenylalanine was required.

Yield 50%. Chromatography: G_3, Rf = 0.4.

Preparation II

0.635 mmoles of tripeptide were coupled as above. Final elution of the product from the Amberlyst column gave some product together with contaminants. This portion was set aside, leaving a yield of the pure peptide of 61.5%.

Preparation III

The general procedure described above was carried out using 0.43 mmoles of the tripeptide.

Chromatography: G_3, Rf = 0.45.

g) Ac-Phe-Leu-Val-His-amide

The boc-peptides from preparation I and III were separately converted to the acetyl derivatives. An accidental loss occurred during an evaporation stage with the first batch. Chromatography G_3 showed a streak with two spots Rf 0.175 - 0.5 when the peptide was applied in dimethyl formamide, but after drying, a single spot was found, Rf 0.14. After recrystallisation from methanol/ether, only 70 mg of first crop product was obtained (20% based on the

tripeptide) with a further 20 mg of second crop material.

The preparation from batch III gave product with Rf in G_3 of 0.14.

Yield 75%, based on the tripeptide. Melting point, 223°-225°C (decomposed from 190°C).

A third preparation of the acetyl peptide was made from the ac-Phe-Leu-Val-His-ester which is described below. The amidination was similar to that used for the tripeptide (section d above).

Yield 100%. Chromatography: G_3, Rf = 0.15.

Melting point 220°-222°C decomposed from 180°C.

The percentage composition of peptides was not very satisfactory. However, the ratios of C : N : H were quite good if a percentage of material which was not analysed (excluding oxygen) was associated with the molecule, particularly if there was also one molecule of water of crystallisation. Although water should not have been present during the synthesis, some moisture in the solvents may have been responsible. The presence of extra "inorganic" material cannot be readily explained and the fairly consistent levels are surprising, considering three independent preparations are being considered. Thus calculated compositions for ac-Phe-Leu-Val-His-amide. H_2O are: C, 58.7%; H, 7.5%; N, 17.1%.

Preparation 1 found C, 51.42%; H, 6.37%; N, 14.19% (12.5% "inorganic" material gives C, 58.7%; H, 7.28%; N, 16.23% - rather low).

Preparation 2 found C, 51.08%; H, 6.69%; N, 14.88% (12.5% "inorganic" material gives C, 58.5%; H, 7.64%; N, 17.04%).

Preparation 3 found C, 54.87%; H, 6.82%; N, 16.14% (6.5% "inorganic" material gives C, 58.8%; H, 7.33%; N, 17.23%.

Kinetic studies were performed before the elemental analyses were complete, and the molecular weight was calculated as representing the free base of the peptide, with no water or "inorganic" matter being accounted for in the weighings. Each batch gave comparable kinetic behaviour in the studies.

h) Ac-Ala-Phe-Leu-Val-His-amide

The material from preparation II of boc-Phe-Leu-Val-His-amide (0.39 mmoles) was used in the synthesis. 230 mg of product was recovered from the Amberlyst column (85%), Rf in G_3 = 0.2 - 0.4. The material appeared pure but after acetylation it was evident that some dicyclohexyl urea and hydroxybenzotriazole were present. Attempts to extract the impurities from an acid aqueous solution, with ethyl acetate were not successful, and so it was decided to apply the product to an Amberlyst 15 column (15 x 2 cm). Some product persistently appeared in the effluent although the capacity of the column should have been adequate.

Product was eluted from the resin with collidine/dimethyl formamide followed by 4% (v/v) triethylamine/dimethyl formamide.

Yield 30% (from commencing purification).

Chromatography: G_3, Rf = 0.091. Decomposed 225°.

% composition calculated for Ac-Ala-Phe-Leu-Val-His amide. H_2O. C, 57.8%; H, 7.45%; N, 17.4%. Two analyses were performed for this sample, giving quite different results: C, 57.70%; H, 6.71%; N, 16.10% and (2) C, 57.35%; H, 7.42%; N, 15.39%. Thus both analyses give rather low estimates for nitrogen and the hydrogen is very low in one, but acceptable in the other. The reason for the discrepancy is not clear, but it is quite likely that a small

amount of carbon and hydrogen containing contaminant was present. The peptide was used without further purification for some kinetic studies, since this may have resulted in insufficient material for any kinetic studies. Thin layer chromatography, electrophoretic behaviour and amino acid analysis suggested the desired product was present.

After evaporating the effluent solution containing product and the two contaminants, an attempt was made to extract the impurities to hot ethyl acetate, in which the product is insoluble. Unfortunately, although this apparently was successful, two Pauly positive spots were detected when the product was subjected to electrophoresis at pH 3.6, 150 kilovolt minutes, and this was not further purified.

i) Ac-Phe-Leu-Val-His-methyl ester

Synthesis of the boc-peptide was on a 0.4 mmole scale, following the general procedure. During elution from the Amberlyst resin, the flow of eluant stopped. Material eluted before the halt was pure, but that eluted after flow was recommenced contained impurities as well as product and was not used further.

Chromatography G_3, Rf = 0.465.

Acetylation proceeded normally to give the required peptide.

Yield 70% (based on the tripeptide).

Melting point 187°-8°C (decomposed from 170°C).

Chromatography: G_3, Rf = 0.39; E_4, Rf = 0.31.

% composition calculated for Ac-Phe-Leu-Val-His-methyl ester. H_2O.: C, 59%; H, 7.48%; N, 14.28%. Found 57.55%. H, 7.04%; N, 13.07%. The slightly low analyses overall and the relatively lower value for nitrogen may be explained by small amounts of laboratory dust etc.

in the sample.

j) Ac-Ala-Leu-Val-His-methyl ester

0.83 mmole of tripeptide ester was coupled to activated boc-alanine. Chromatography: G_3, Rf = 0.48. Yield 77% . Acetylation using the Amberlite 1R4B column for exchange of the trifluoroacetate ion (cf. original preparation, section a above), gave the required product.

Yield 65% (based on the tripeptide).

Chromatography in G_3, Rf = 0.144; E_4, Rf = 0.192.

% composition, calculated for ac-Ala-Leu-Val-His-methyl ester. H_2O. C, 53.8; H, 7.8%; N, 16.35%. Found C, 53.52%; H, 7.43%, N, 15.66%.

k) Preparation of ac-Ala-Leu-Val-His-amide

This was prepared from 0.41 mmole of the tripeptide amide, according to the general methods.

Yield 40% (based on the tripeptide)

Chromatography Boc-Ala-Leu-Val-His-amide, G_3, Rf = 0.35.

Ac-Ala-Leu-Val-His-amide, G_3, Rf = 0.05; E_4, Rf = 0.02.

SECTION 5 ENZYME PURIFICATIONS

5.1 Separation of human pepsin and gastricsin by chromatography on Amberlite 1RC50

The enzymes were isolated from gastric juice by the method of Tang (1970), using stepwise pH elution from Amberlite CG-50 resin, with 0.2 M citrate buffers. For reasons which have not been resolved, the pepsins did not behave on our column in the way detailed by Tang, the pepsin being eluted in the pH 3.8 buffer which Tang had used as a wash. The gastricsin was also eluted at a slightly low pH value compared with Tang's data, but in the same buffer. During a further purification stage, much of the gastricsin was unfortunately lost, and another preparation was made as detailed below.

Gastric juice was obtained over a period from the Royal Infirmary, Edinburgh and was filtered to remove any solid material, dialysed against several litres of distilled water and freeze dried. 3 g of freeze dried material (from about 2 l gastric juice) was dissolved in about 30 ml of 0.2 M citrate buffer, pH 3.0 and centrifuged in a Spinco centrifuge at 30,000 rpm for 30 minutes. The supernatant was applied to a 5.5 cm x 30 cm column of Amberlite CG50 which had been prepared as described in section 2.3, and equilibrated with pH 3.0 citrate buffer. The chromatography was performed at room temperature (23°C), 50 ml fractions being collected on a Central fraction collector and the eluate being monitored with a Gilson ultra-violet recorder. The flow rate was approximately 1 ml per minute. After washing with 0.2 M citrate

buffer, pH 3.0 an additional buffer, not used by Tang, at pH 3.4 was applied as a wash. Buffers of pH 3.8, 4.2 and 4.6 were applied in order, about two litres being required for the eluate to attain the pH of the applied buffer.

The absorbance at 280 nm of fractions over the peak regions were measured in a Unicam SP500 spectrophotometer, and samples were assayed for haemoglobin digesting activity by the method of Tang (section 2.1a). An elution profile is shown in figure 5. Fractions were pooled as indicated. 80% of the total activity applied to the column was recovered in the two pepsin peaks. Polyacrylamide gel electrophoresis (section 2.5) performed at this stage indicated that the pepsin was apparently pure, but that the gastricsin had slight contamination with pepsin A. The pepsin was concentrated and washed using a Diaflo ultrafiltration cell and freeze dried , yielding 250 mg protein; specific activity 39.5

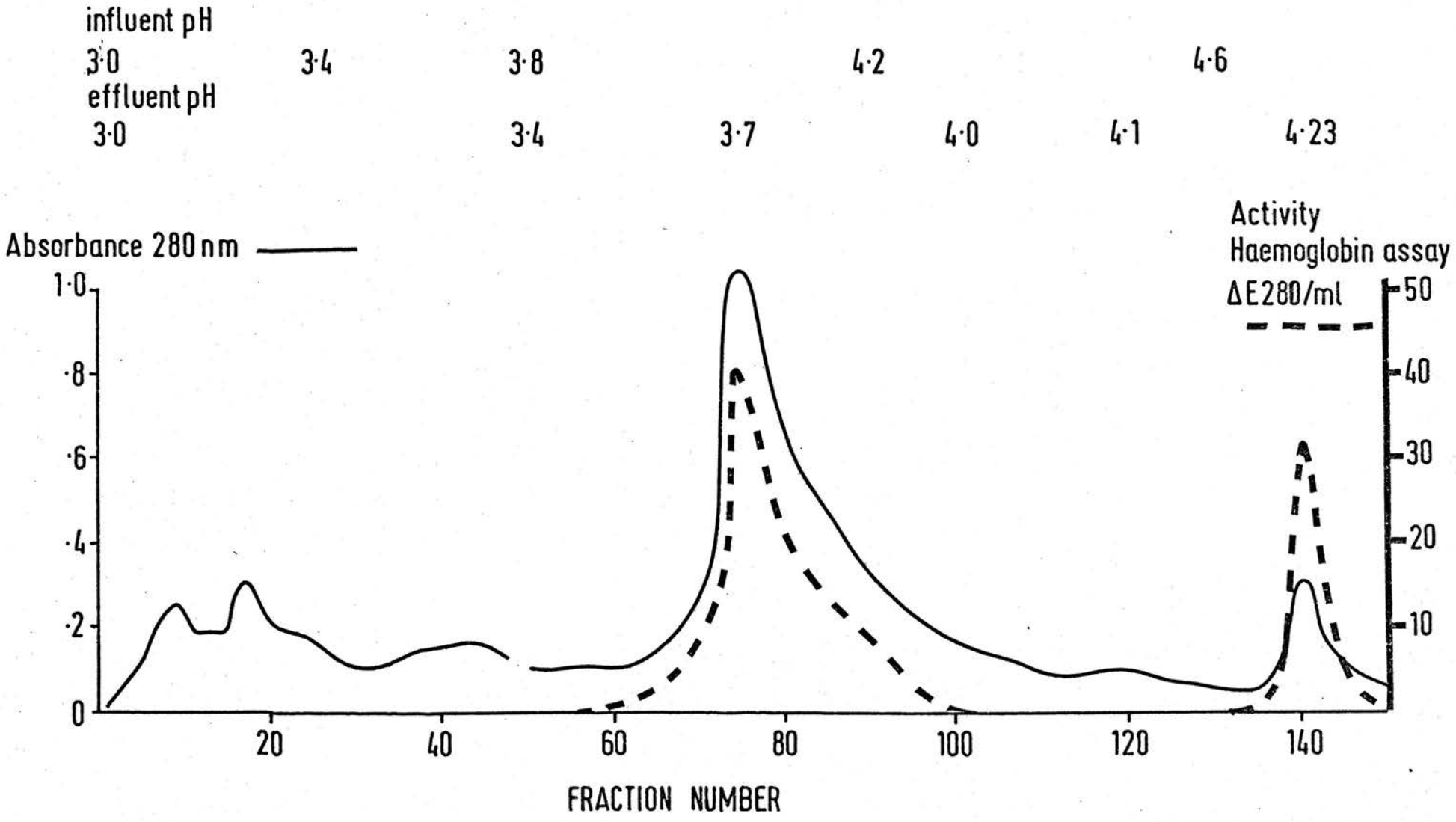
$$\left[\frac{\Delta \text{E280/ml}}{\text{E280}} \right]$$

5.2 Chromatography of gastricsin on DEAE cellulose

The pooled gastricsin fractions were concentrated in a Diaflo cell to 40 ml and applied to a DEAE cellulose column (2.5 x 17 cm) which was equilibrated with 0.1 M acetate buffer pH 4.0. The gastricsin solution was allowed to flow through the column which was then washed with 100 ml of the acetate buffer. An exponential sodium chloride gradient (using a 500 ml mixing vessel) was applied (0 - 0.2 M), in 1 M acetate buffer, pH 4.0. 25 ml fractions were collected. An elution profile is shown in figure 6, together with

FIGURE 5

CHROMATOGRAPHY OF HUMAN GASTRIC JUICE ON AMBERLITE C.G.50 0·2M CITRATE BUFFERS. 50ml FRACTIONS



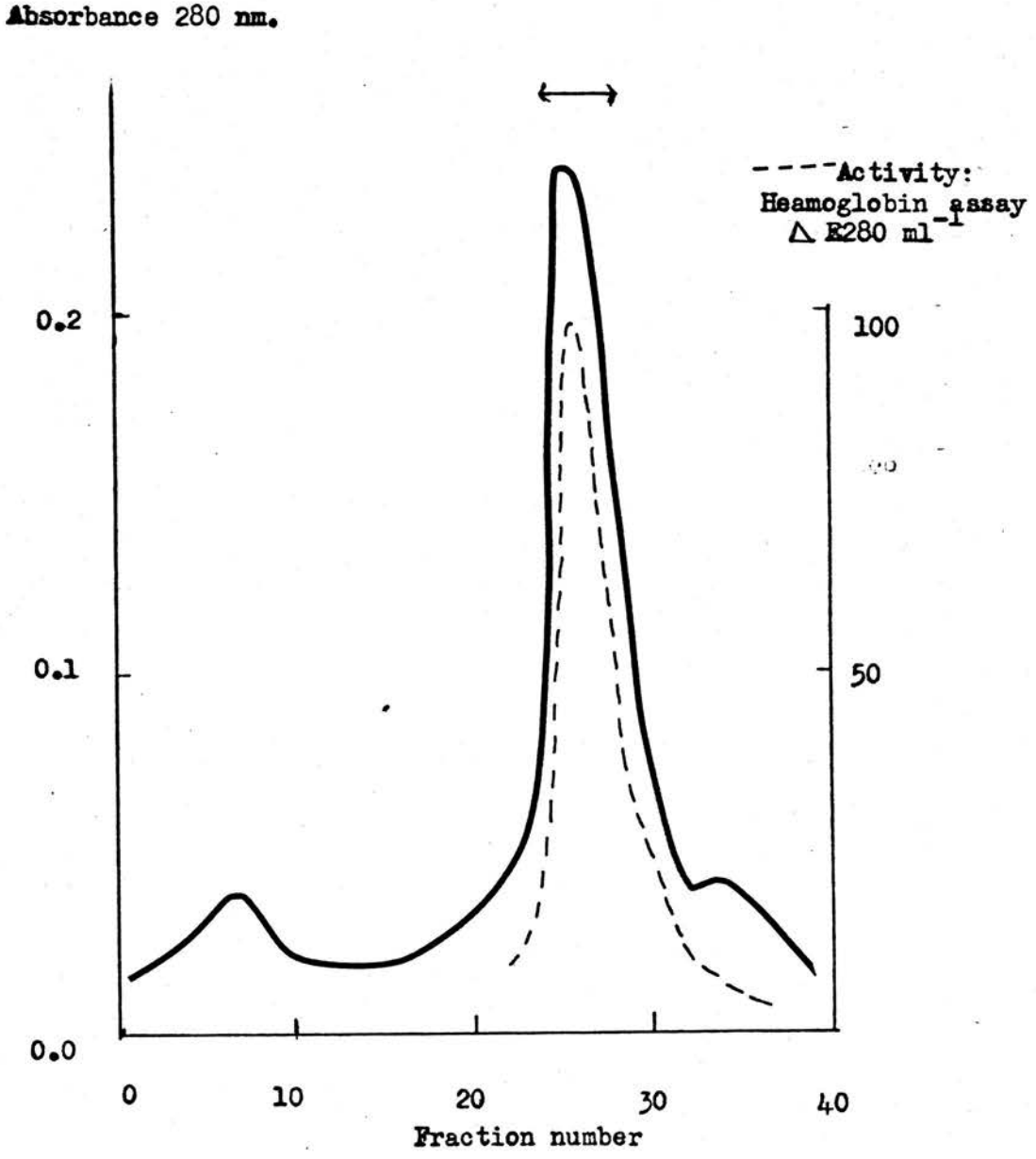


FIGURE 6 Chromatography of gastricsin on DEAE cellulose.

NaCl gradient in 0.1 M acetate buffer pH 4.0

25 ml. fractions.

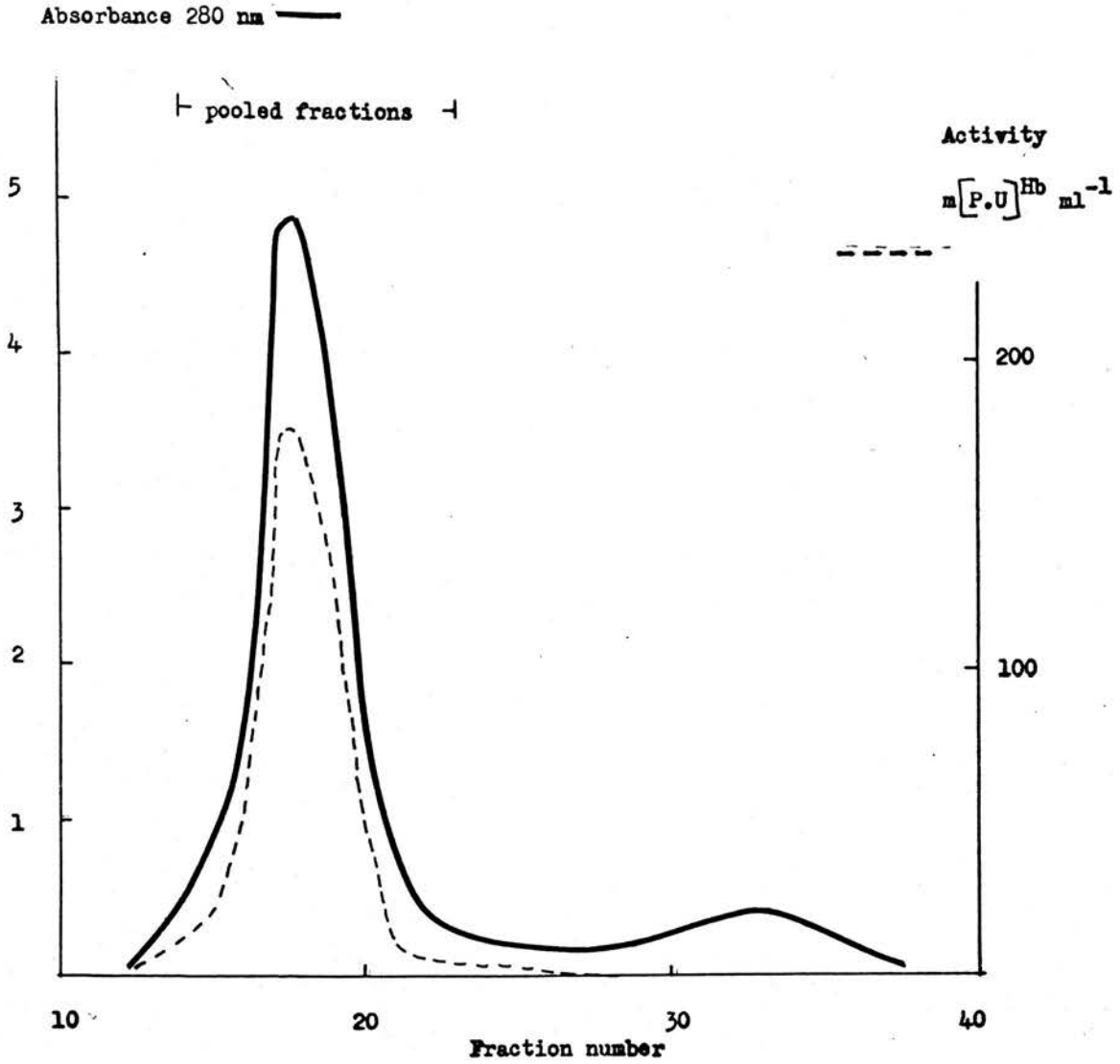


FIGURE 7 Chromatography of pepsin C on G 100 Sephadex, in 0.1 M. Acetate buffer pH 5.6 ; 6.15 ml fractions.

the haemoglobin digesting activity of the enzyme. The fractions indicated were pooled, concentrated and washed, & the solution stored frozen.

50% of the activity applied to the column was recovered; the specific activity measured as $\frac{\Delta E280/ml}{E280}$ in the haemoglobin assay of Tang was 85.

5.3 Chromatography of porcine pepsin C on Sephadex G100

Preliminary studies with the enzyme which had been previously prepared (section 2.7) showed that it had a high 'blank' value when used in a system with ninhydrin as the monitoring reagent. It also had rather a low specific activity $\frac{(30m[PU]^{Hb}perml)}{E280}$ compared with a value of 35 - 36 normally obtained for a fresh preparation. Some degradation of the enzyme had probably occurred on storage, and it was therefore decided to rechromatograph the pepsin C by gel filtration, to remove small fragments.

100 mg of pepsin C were dissolved in 5 ml 0.1 M acetate buffer pH 5.6. The solution was applied to a 2.5 x 20 cm column of G100 Sephadex, equilibrated with the same buffer. 6.15 ml fractions were collected and the ultraviolet absorbance of the effluent was monitored. The absorbances at 280 nm and haemoglobin digesting activities (by the method of Ryle; section 2.1a) of the relevant tubes were measured and shown in figure 7. The fractions indicated were pooled and dialysed against distilled water. Most of the material was kept in the frozen state. 76% of activity applied to the column was recovered, and the specific activity was found to be 35.7 $\frac{m[P.U]^{Hb}/ml}{E280}$. The high blank value of the enzyme

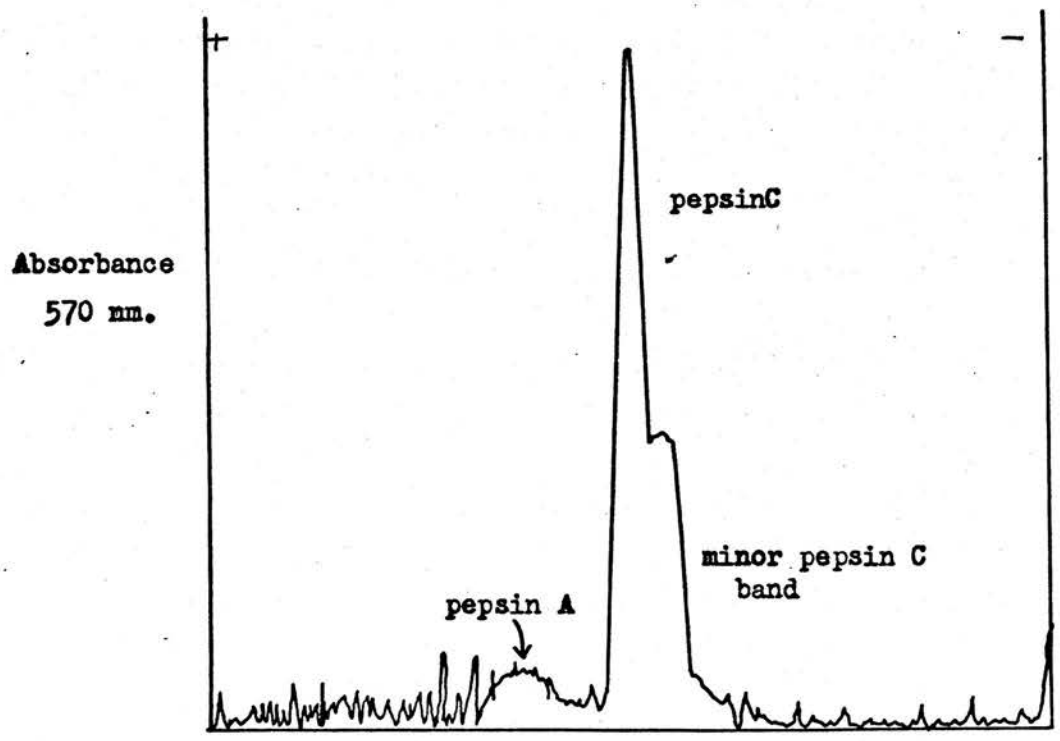


FIGURE 8 Polyacrylamide gel electrophoresis of pepsin C stained for protein. Gel scan at 570 nm.

in ninhydrin monitored assays was also markedly reduced.

5.4 Polyacrylamide gel electrophoresis of the pepsin preparations

The electrophoreses were carried out as described in section 2.5, and for each enzyme a Coomassie blue protein stain and an activity stain were performed.

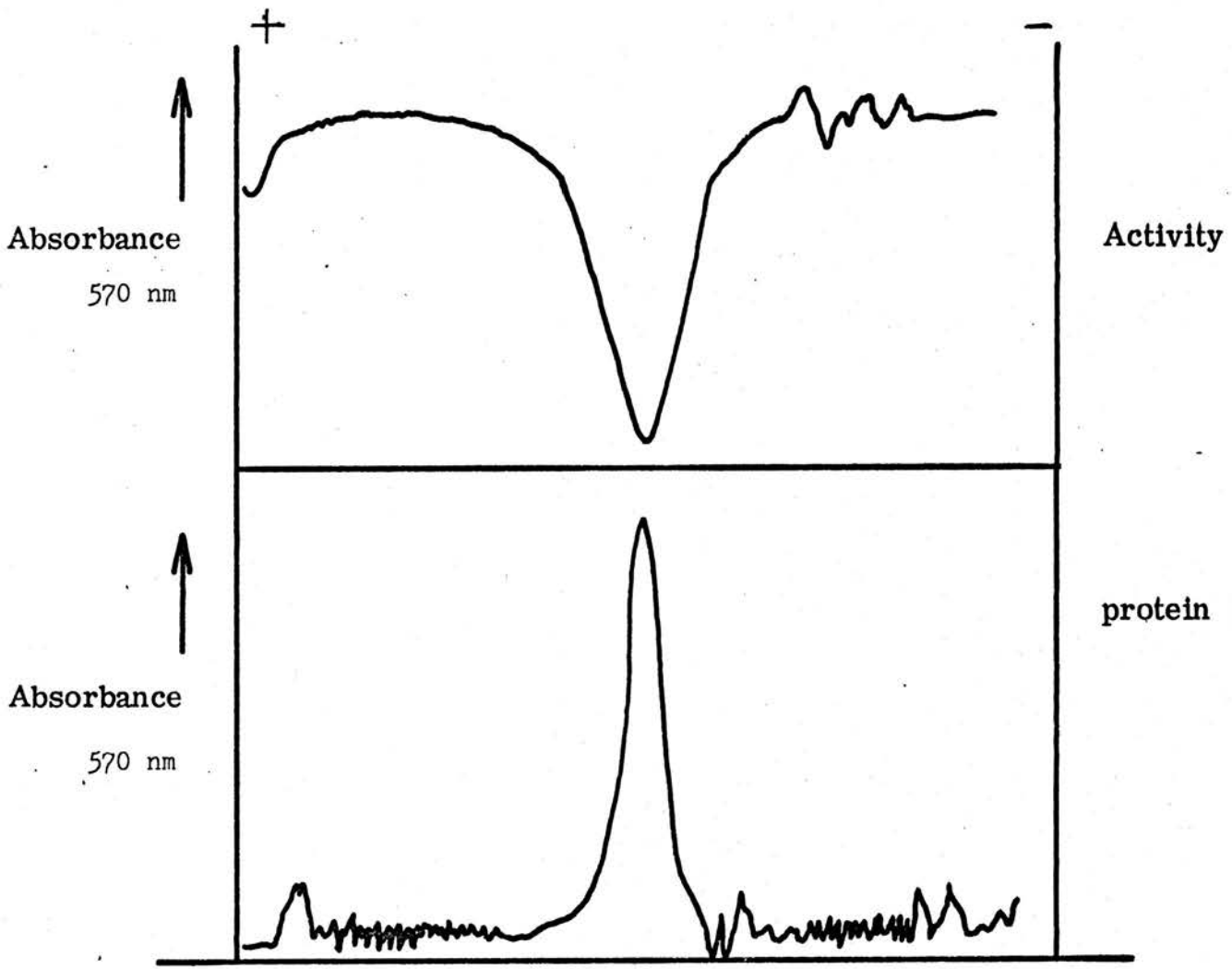
a) Porcine pepsins

Pepsins do not stain with Coomassie blue very readily, but it was evident visually that pepsin A was slightly contaminated with pepsin C and that some protein with the same mobility as pepsin A was present in the pepsin C preparation. The pepsin C appeared to be present as a double band. A scan of a typical gel of the pepsin C preparation at 570 nm, and stained for protein, is represented in figure 8. The amount of pepsin A present is evidently quite small, the peak almost being lost in the base line. However a similar gel subjected to the activity stain indicates sufficient enzyme is present to digest all the haemoglobin present under the conditions used. The double band representing pepsin C may be due to the presence of a minor variant to the extent perhaps of 20%. Although pepsin B has a similar mobility to pepsin C under the conditions used, the method of preparation of pepsin C from pepsinogen C makes it unlikely that this enzyme is causing contamination, since elution of the two zymogens in the preparation are widely separated. It is worth noting that in the preparation of pepsin B, double bands have been observed by Ryle (1965).

b) Human enzymes

The gastricsin appears to be homogeneous when subjected to

FIGURE 9 Polyacrylamide Gel electrophoresis of Gastricsin



electrophoresis on polyacrylamide gels, which were stained for both protein and activity. Gel scans at 570 nm are represented in figure 9.

The pepsin showed quite broad bands of enzyme after both protein and activity staining on the gels. However, the enzyme appeared free from contamination.

5.5 The pepsin impurity of pepsin C

In previous preparations (e.g. Ryle & Hamilton 1966), it has been observed that a ratio of the specific activities measured as μmoles diiodotyrosine produced per minute per ml in the activity towards acetyl phenylalanyl diiodotyrosine to $[PU]^{Hb}$ in the haemoglobin assay is 0.2 for pepsin C and 7 for pepsin A. This represents a contamination of about 3% in normal preparations, assuming that pepsin C has no intrinsic activity towards the synthetic substrate. The pepsin C used in these studies gave a ratio of 0.67, representing a 9% contamination with pepsin. This value is rather high but assays which have been performed on the substrate ac-Phe-Leu-Val-His-amide, which has been used most for the kinetic studies described later, show that the pepsin A contributes at most, only 1% of the activity towards this substrate.

5.6 The activity of porcine pepsin A

The preparation showed a low specific activity in the haemoglobin assay, having only 75% of the activity which it had when prepared. However as it was only to be used in minor comparative studies with the synthetic substrates, no further purification was carried out.

5.7 Estimation of enzyme concentration

Enzyme concentrations were determined by measuring the absorbance of a solution of the enzyme in water or buffer at 280 nm and relating the absorbance to that of a solution of known concentration. For porcine pepsin C and porcine and human pepsins A, a solution containing 1 mg ml^{-1} of freeze dried enzyme gave an absorbance of 1.3 units. A similar value was assumed for gastricsin which was kept in solution after the final purification rather than being freeze dried.

In order to determine k cat values, an estimation of the molar concentration of the enzyme is required. Values of the molecular weight which have been used are 36,000 (Ryle & Hamilton 1966) for pepsin C and 34,500 for pepsin A. Values quoted by Tang (1970) of 31,400 for gastricsin and 34,000 for pepsin were used for the human enzymes.

SECTION 6. KINETIC STUDIES WITH PEPSIN C

6.1 Use of the Technicon Autoanalyser II

For the majority of the kinetic studies performed, the hydrolysis of a substrate by one of the enzymes was monitored continuously using a Technicon autoanalyser II. This method was introduced into the study of pepsin catalysed reactions by Lenard *et al.* (1965) and Cornish-Bowden and Knowles (1965) and depends on monitoring the generation of a free amino group in the hydrolysis of a peptide bond, by a colour formation with ninhydrin reagent. The initial velocity of the reaction is estimated from the continuous trace. A flow scheme for the instrument is shown in the diagram, figure 10. Table 3 gives details of the pump tubing etc.

The Technicon system has several advantages over a manual estimation, when initial velocities are required. A continuous trace is obtained on the early part of the reaction, but it is difficult to obtain very many results during the first few minutes of a reaction when a manual estimation is performed. Errors in pipetting, timing etc. and instrument limitations reduce the accuracy of a manual estimation, but continuous sampling and identical processing through the automatic system allows higher sensitivity towards slight changes in absorbance, which might not be recorded in a manual experiment.

Ninhydrin Reagent:- 5 g ninhydrin (Koch-Light) were dissolved in 183 ml 2-methoxy ethanol (Shell), through which nitrogen had been bubbled. 0.5 g hydrindantin (BDH) was added and the solution bubbled with nitrogen for 5 minutes. 88 ml 4 M sodium acetate

TABLE 3

Description of the pump tubing used for the Technicon Auto-Analyser

Line	internal diameter (inch)	flow rate (ml. min^{-1})	type of tubing
Nitrogen	0.03	0.32	standard
Ninhydrin	0.035	0.42	solvaflex
Substrate	0.035	0.42	standard
Return (from colorimeter)	0.025	0.23	solvaflex

Solvaflex transmission tubing was used for connections.

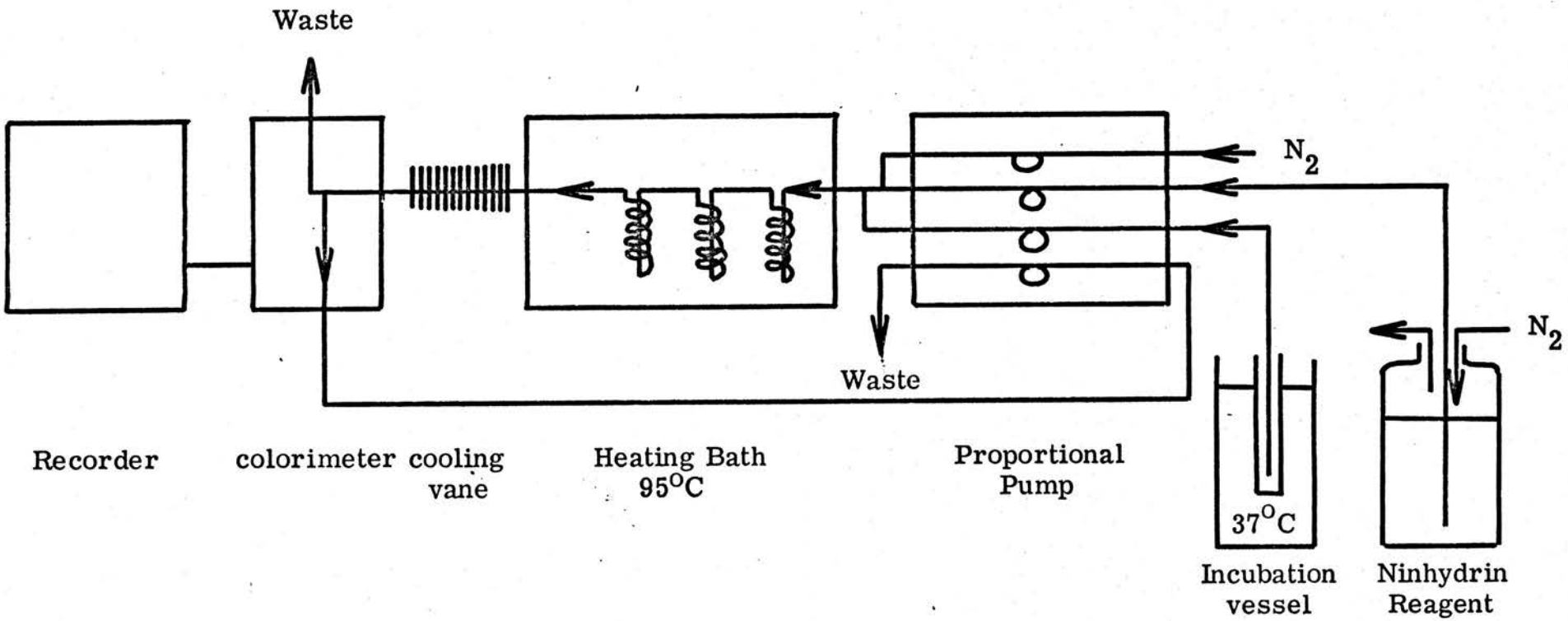


FIGURE 10 Flow Scheme for continuous monitoring of enzyme reaction by Technicon Auto-Analyser.

buffer, pH 5.5 were added and nitrogen again bubbled through the solution. 750 ml of a 50% (v/v) aqueous solution of 2-methoxy-ethanol which had been bubbled with nitrogen were added, and nitrogen bubbling continued for a further 5 minutes. The reagent was stored in an amber bottle under an atmosphere of nitrogen.

The instrument was calibrated, using 0.05 M citric acid in the sample line to zero the recorder, and 0.04 or 0.1 mM leucine in 0.05 M citric acid, as appropriate, to give full scale deflection.

In a typical assay, 40 µl of enzyme solution was added to 4 ml substrate solution which had been brought to 37°C. After stirring, the probe was introduced, and sample was continuously drawn by the pump to be mixed with ninhydrin reagent which had been segmented with nitrogen bubbles. The ninhydrin reagent stops the reaction, and reacts with any free amino groups present, those present in the enzyme, and those produced by the hydrolysis of a peptide bond. The blue colour produced by heating the solution in an oil bath at 95°C for about 15 minutes was monitored by the colorimeter at 570 nm and a trace was produced by the recorder. This represented about 9 minutes of reaction. An enzyme blank was performed using buffer in place of substrate to determine the amount of colour produced due to the enzyme, and to detect any measurable amount of autodigestion which took place over the period. This could then be subtracted from the estimation of the rate of substrate hydrolysis, if necessary. Examples of traces of a standard sample and an initial velocity experiment are shown in figure 11.

A lag of about 70 seconds between addition of the enzyme and mixing with ninhydrin occurs, which causes a step increase at the

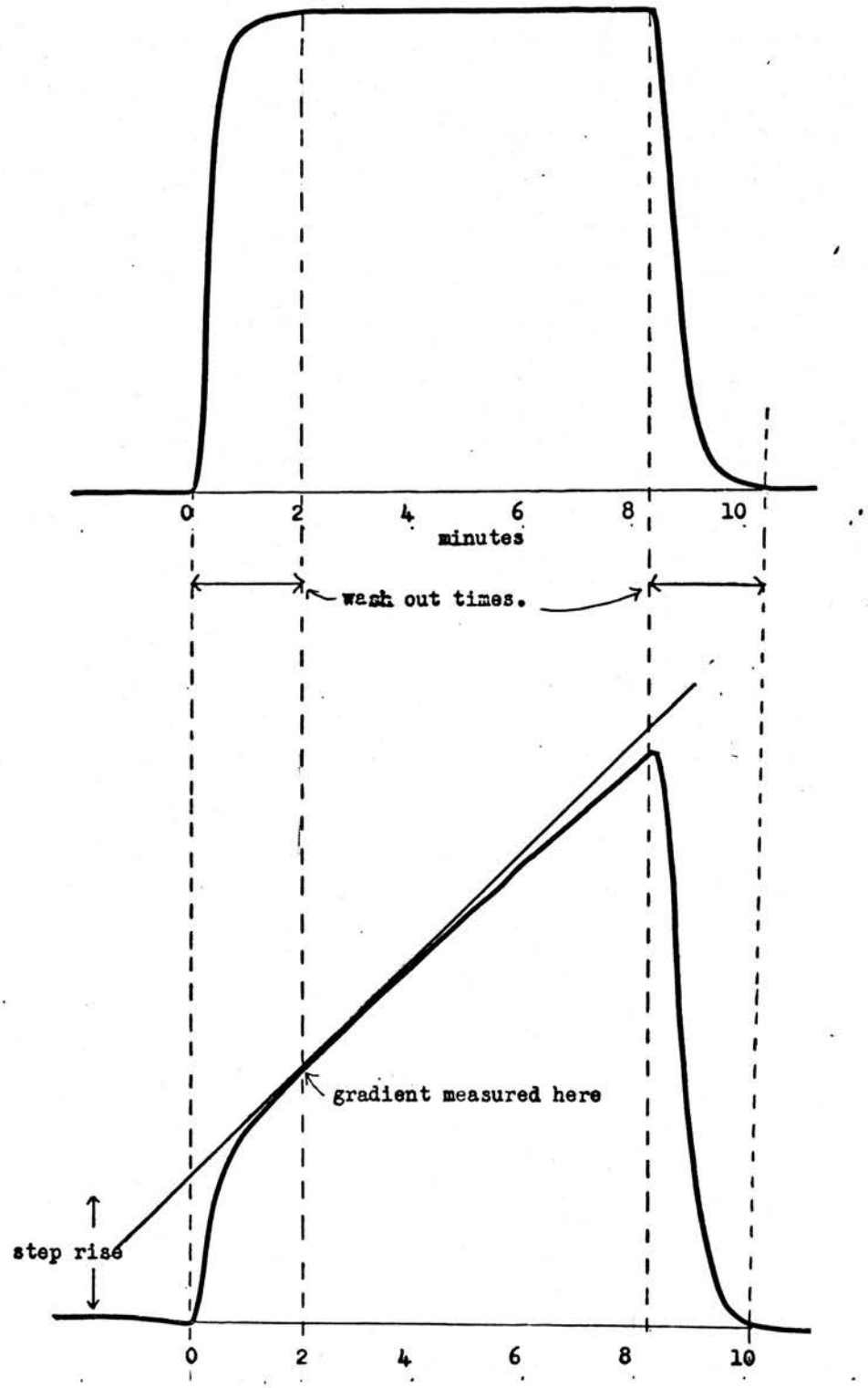


FIGURE 11 Examples of traces from the Auto analyser (a) standard trace, (b) experimental trace, showing the effect of the wash out time and the 'step rise', caused by the delay between adding enzyme to the substrate and the reaction being stopped by ninhydrin reageant.

beginning of the trace. This represents the digestion which has occurred in the sample tube before the reaction could be stopped by the ninhydrin reagent. A sudden change in the potential colour formation in the sample requires a certain amount of time for full colour development to be realised. This is due to the nature of the flow in the sample tube before bubble segmentation is introduced, after the pump, which then prevents mixing between short sections of the sample. A 'wash out' time is therefore seen on the trace, at both the beginning and end of a sample, and may be most clearly seen in the figure representing a standard trace. A gradual approach to the true level is observed over a period of 1.5 - 2 minutes. Thus the early curvature of the trace for an initial velocity experiment must be observed cautiously to ensure that the curvature due to the wash out time is not included in the estimation of the rate of hydrolysis. It can be seen therefore that the most accurate region of the trace commences after about 2.5 minutes of reaction have already taken place. Slight irregularities in the bubble pattern which may occur during the flow through three coils in the heating bath are difficult to avoid, and probably are responsible for a little unevenness in the trace. Also, the inevitable introduction of an air bubble into the sample tube, when the probe is placed in the reaction medium sometimes caused slight bumps in the curve.

6.2 Estimation of the initial velocity

The initial velocity of a given hydrolysis reaction was estimated by drawing a tangent to the trace at the earliest point, after which the slope of the trace could be considered to be

entirely due to increasing product formation. In most cases, the first portion of the trace was in fact linear and thus the errors in using the method were probably quite low. It should, however, be noted that the velocity at time 2.5 minutes rather than at time zero was actually recorded, although under conditions where linearity is observed for several minutes, the two values should be similar.

An attempt was made to determine the velocities mathematically, by fitting a polynomial equation to the data. The second constant in the equation $a + bx + cx^2 + \ldots$ would give the slope required. However, since the trace was short, only ten or twelve data points could be used, these being read from the trace, and results obtained were not satisfactory. A change in the first data point of 1%, when the width of the line itself covered 4% of the value, sometimes resulted not only in a great change in the calculated velocity, but also in the 'best-fit' order of polynomial which had been assigned. Small irregularities in the trace would therefore also lead to incorrect estimates. Since the exact time zero was not known relative to the first data points on the graph, this method could still only give the velocity at approximately 2.5 minutes from the start of the reaction. Therefore although this method would be less subjective than the one used, more sophisticated data handling is required for satisfactory results. For example, more data could be generated using a data logging device giving many accurate readings at short time intervals, or alternatively the reaction could be studied over a longer period. This however would be expensive in terms of the substrate required. A true initial velocity could be obtained if the start of the reaction was very

accurately recorded, and the points from the data logger corresponded to exact known times of the reaction. These values could then be used to extrapolate the curve to zero time. The large number of points obtainable by a data logging device would average out the irregularities in the trace. No data logger was available, however.

6.3 Estimation of the kinetic parameters Km, V max

a) Experimental

For each estimation a series of traces was obtained, representing the initial rate of hydrolysis at at least five substrate concentrations. The range of substrate concentration was as wide as both the solubility and availability of the substrate would allow. In most cases the estimated value of Km lay in, or a little above, the range of concentrations used, but some experiments with higher substrate concentrations would have been desirable.

As far as possible, similar enzyme concentrations were used throughout, to avoid too many dilution errors, and to obtain ready comparison between the parameters obtained for different substrates, or under different conditions. Two different instrument calibrations were used, according to the substrate, so that gradients of a suitable magnitude for measuring were obtained. Stock solutions of enzymes were kept frozen in small batches, each being sufficient for about one week's supply. A batch was thawed and diluted to give enough aqueous enzyme solution for one or two days' experiments, this solution being kept at $4^{\circ}C$. The few freeze-thaw cycles for each batch did not affect the enzymic activity and it was felt

more satisfactory than subjecting large volumes of enzyme solution to repeated freezing and thawing as this could cause some denaturation of the protein.

b) Evaluation

The kinetic parameters Km and V max were estimated by means of the direct linear plot described by Eisenthal and Cornish-Bowden (1974). The advantages of this method are discussed in this paper and in a subsequent one by Cornish-Bowden and Eisenthal (1974). A similar method has been described by de Miguel Merino (1974). Used graphically it is a simple method to apply, as no calculations are required on the data or on the given results. Thus straight lines joining corresponding values of substrate concentration (on the minus x axis) and estimated initial velocities (on the y axis), give series of intersections in the positive quadrant, the median of which gives the value of V max and Km. The values of Km and V-max may be estimated accurately by computation. In the linear transformations, weighting is necessary to avoid bias in a linear regression calculation, towards experimental points which are likely to be less reliable. Since the median, rather than the mean is used in the calculation of Km and V max by the direct linear plot, the method does not allow unfair contribution from an outlier but neither does it reject it subjectively. Calculation of a slope by 'least squares' analysis may take more account of a single bad point than of several accurate ones, giving a poor estimate of the parameters.

In some of the studies to be described graphical representations of results using linear transformations will be shown, as these are

useful for illustration, for example, when Michaelis Menten kinetics are not obeyed, and in inhibition studies, where the types of inhibition are more easily recognisable, and have been defined with reference to a linear plot (Cleland 1963).

The rate constant kcat is the value $\frac{Vmax}{Eo}$ s^{-1}, where Eo is the total enzyme concentration.

Throughout the work, owing to the time and materials available, the emphasis has been on a superficial exploratory level, using relatively little data, rather than as a rigorous kinetic survey of the enzymes. For example, in the pH dependence studies, a single estimation of the kinetic parameters was made over a wide range of pH values, rather than performing duplicate studies at only half the number of pH values. Thus although in some cases the errors might be quite large, the overall behaviour of the enzymes towards their substrates may be observed fairly reliably. When single experiments have been performed for estimations of Km and Vmax, some cross reference (for example to preliminary studies) was made to ensure that the enzyme used was fully active.

6.4 Study of the suitability of synthetic peptides as substrates for pepsin C

Peptides were tested by performing a manual estimation of the time course of a reaction, using a fairly high enzyme concentration (0.1 mg ml^{-1}), or directly with the Technicon autoanalyser system. The conditions were 37°C and pH 2.07 and the substrate concentration was up to 0.5 mM. In the Technicon system, readily digestible peptides were found to give an appreciable slope when

the final enzyme concentration was 2 - 3 $\mu g\ ml^{-1}$.

Preliminary investigations

The peptide which was first synthesised, ac-Ala-Leu-Val-His-methyl ester showed very slow digestion with pepsin C, but the results were not very satisfactory, partly because of a very high enzyme blank, with some autodigestion taking place. The enzyme was rechromatographed for subsequent work (see section 5.3). Some deesterification of the peptide seemed to have occurred, and it was not studied further at this stage. It was decided to prepare some ac-Tyr-Leu-Val-His-methyl ester in the hope that the aromatic group would make the peptide more susceptible to hydrolysis. A second preparation of the alanine peptide was also made (section 4.4.j).

Initial studies with the protected tyrosine peptide indicated that rapid hydrolysis occurred exclusively at the tyrosyl-leucyl bond, as shown by separation of the hydrolysis products by electrophoresis (described below). Attempts were made to determine the kinetic parameters for the peptide, but considerable deesterification of the substrate had taken place. The reason for this was not clear but reesterification using dicyclohexylcarbodiimide and hydroxybenzotriazole in methanol was not thought advisable, since racemisation of the histidine residue was likely. The ester was separated from the free acid (section 4.4.c(I)) and a few studies were done with the latter compound. It was decided to prepare some more peptides involving phenylalanine and alanyl-phenylalanine, and an amide group would be used to protect the carboxyl terminus as it should be less labile than the ester group. A

further preparation was also made of the acetyltyrosyl-peptide ester, taking care to avoid the presence of excess base at any stage of the synthesis, as the deesterification may have been due to a little base being present in the substrate, despite purification. No elemental analysis was performed on the first preparation of the peptide so this possibility cannot be ruled out.

The peptides which have been prepared as potential substrates for pepsin C are listed below:-

ac-Ala-Leu-Val-His-methyl ester

ac-Ala-Leu-Val-His-amide

ac-Tyr-Leu-Val-His-methyl ester

ac-Tyr-Leu-Val-His-amide

ac-Phe-Leu-Val-His-methyl ester

ac-Phe-Leu-Val-His-amide

ac-Ala-Phe-Leu-Val-His-amide

The synthesis of these peptides has been described (section 4.4).

6.5 Electrophoresis of products of prolonged incubations of the substrates with pepsin C

Samples from overnight incubation mixtures of the substrates with pepsin C were subjected to paper electrophoresis at pH 2 and 3.6 (section 2.4). The products Leu-Val-His- as the free acid, ester or amide were readily located with both ninhydrin and Pauly stains, and acetyl tyrosine could also be identified with Pauly reagent. Acetylphenylalanine, acetyl alanylphenylalanine and acetyl alanine were less easy to locate as the chlorine method did not give reproducible results.

The tyrosine peptides showed only two product spots, corresponding to acetyl tyrosine and a Leu-Val-His peptide by comparison with authentic samples, and a total disappearance of substrate. Thus hydrolysis is exclusively at the Tyr-Leu bond.

Only a single spot corresponding to a Leu-Val-His peptide was located for the other peptides, suggesting a single cleavage point in these peptides also. This was confirmed for ac-Phe-Leu-Val-His amide, by eluting products of an incubation mixture which had been subjected to electrophoresis. High loading of an authentic sample of acetyl phenylalanine followed by the chlorine detection method indicated the locality of one presumed product, and a marker strip stained with ninhydrin, showed the position of Leu-Val-His amide. Hydrolysis of the eluate from different regions followed by amino acid analysis showed only phenylalanine in one fraction, and leucine, valine and histidine in equimolar amounts in another. No amino acids were significantly represented in any other portion of the electrophoretogram, including the fraction which would represent any undigested substrate.

Both ac-Ala-Leu-Val-His-methyl ester and the corresponding amide required very high concentrations of enzyme (0.5 mg ml^{-1}) for hydrolysis to be readily detectable after overnight incubation, and so these peptides were not suitable for further kinetic studies.

6.6 Kinetics of the hydrolysis of synthetic peptides with pepsin C

Kinetic analyses using the Technicon Autoanalyser were initially performed at substrate concentrations in the range 0.1 - 0.5 mM, this range being extended if the Km seemed to be much beyond the

TABLE 4

Kinetic parameters for the hydrolysis of synthetic peptides by Pepsin C at pH 2.07 and 37°C

Substrate*	Range of So (mM)	Km app (mM)	k cat (s^{-1})	k cat/Km ($s^{-1}mM^{-1}$)
ac-Tyr-Leu-Val-His-OMe	0.025 - 0.1	0.21	1.1	5.24
	0.25 - 1.0	2.68	10.1	3.77
ac-Tyr-Leu-Val-His-OH	0.1 - 1.0	0.8	1.1	1.38
ac-Tyr-Leu-Val-His-NH_2	0.1 - 0.5	0.82	1.09	1.34
	1.0 - 2.0	5.2	5.26	1.01
ac-Phe-Leu-Val-His-OMe	0.1 - 0.7	1.37	4.10	2.99
ac-Phe-Leu-Val-His-NH_2	0.5 - 1.5	1.72	6.05	3.52
ac-Ala-Phe-Leu-Val-His-NH_2	0.075 - 0.375	0.7	7.54	10.16

So - Initial substrate concentration

Km app - apparent Km

* Hydrolysis occurs exclusively at the amino side of the leucyl residue.

highest substrate concentration tested. However, the low solubility of some of the substrates and the wish to conserve the available peptide material did not always allow the range of substrate concentrations to be as wide as would have been desirable.

The parameters which have been estimated for hydrolysis of some of the peptides with pepsin C are shown in Table 4, which also shows the range of substrate concentration used. Hydrolysis was exclusively at the aminoterminal side of leucine.

a) Hydrolysis of tyrosine containing peptides

It can be seen from the Table that two sets of values of kinetic parameters were obtained for two of the peptides, depending on the range of substrate concentration used. Plots of v against S over the entire concentration range tested for ac-Tyr-Leu-Val-His-methyl ester and the corresponding amide, did not give a simple hyperbola, and thus no unique values of the parameters were obtainable. The data seemed to fall into two distinct groups, and details of the experiments will be given in a later section. Thus although these peptides were readily digested, the ester particularly having a promising value of kcat/Km, when using low substrate concentrations, they were felt to be unsuitable for basic informational studies such as pH dependence and inhibition studies. The carboxyl group on the free acid peptide would complicate studies in the pH range 3 - 5, where the group would be partly ionised.

b) Hydrolysis of phenylalanine containing peptides

Initial studies with ac-Phe-Leu-Val-His-amide in the range 0.1 - 0.5 mM gave a series of parallel lines in the direct linear plot (i.e. the initial velocity is directly proportional to the

substrate concentration) kcat/Km can therefore be estimated independently over this range from the gradient of a plot of v against S. The value obtained is 3.6 s^{-1} mM^{-1}, agreeing well with that obtained from the individual parameters determined over a wider concentration range (3.52 s^{-1} mM^{-1}).

Ac-Phe-Leu-Val-His ester appeared to give complex data similar to two of the tyrosine peptides described above and so was not studied further.

Ac-Ala-Phe-Leu-Val-His-amide was found to be very insoluble, preventing kinetic studies at concentrations above 0.375 mM. Thus despite its favourable kcat/Km value, it was not used for detailed kinetic studies. For the study with this peptide, a solution which was nominally 0.5 mM in 0.05 M citric acid was found to contain some undissolved material. This was removed by centrifugation and the supernatant was also taken for hydrolysis and amino acid analysis (section 2.6) which showed that 75% of the substrate had dissolved, allowing the determination of the exact concentrations of substrate which had been used.

In general it can be seen that phenylalanyl-leucyl bonds are more rapidly cleaved than are tyrosyl-leucyl bonds. The extension of the peptide ac-Phe-Leu-Val-His-amide by the insertion of an alanyl residue at the N terminus results in more rapid cleavage of the susceptible bond. If the Km is considered an inverse of the binding of the peptide to the enzyme, the tyrosyl peptides seem to be more tightly bound than are the phenylalanyl peptides, and the ester derivatives more so than the amides. The addition of an alanyl residue apparently increased the binding considerably,

although whether this is necessarily a factor in the increased rate of hydrolysis of the peptide is not clear, (as discussed in the introduction with reference to Fruton's work with pepsin A).

In summary, the hydrolysis of peptides of the type ac-X-Leu-Val-His-Y increases as X is alanyl, tyrosyl, phenylalanyl and alanyl-phenylalanyl, with Y being a methyl ester or an amide protecting group.

The peptide used for most further studies was ac-Phe-Leu-Val-His-amide. The Km was rather high (1.72 mM) and substrate concentrations to this level were not used, but the kcat/Km ratio was quite favourable. No complex effects were observed in the concentration range studied but it is possible that if rates at very much higher substrate concentrations had been performed, similar effects to those described for two of the tyrosine peptides and ac-Phe-Leu-Val-His-ester might have been observed.

6.7 pH dependence of the hydrolysis of ac-Phe-Leu-Val-His-amide with pepsin C

The kinetic parameters of the hydrolysis of ac-Phe-Leu-Val-His-amide were determined in the pH range 1.0 - 5.5 using substrate concentrations between 0.5 and 1.5 mM. Citrate buffers (0.05 M with respect to total citrate ion) were used to control the pH of the system between pH 2.0 and pH 5.5. Below pH 2.0 the pH was achieved using hydrochloric acid, although the final solution was still 0.05 M with respect to citrate. The pH of the solutions were measured at 37°C and the concentration of each form of citrate ion was calculated using a computer programme. Sodium

TABLE 5

Composition of the citrate buffers used for the pH dependence studies (sections 6.7, 6.8)

50 ml 0.1 M citric acid and x ml 0.2 M sodium hydroxide were made up to 100 ml with distilled water. Ionic strength of 0.175 M was achieved by addition of NaCl. For pH values below 2, HCl was added.

pH at 37°C	ml 0.2 M NaOH /HCl	I (M)	g.NaCl added
1.0	50.0	0.100	0.439
1.3	25.0	0.051	0.730
1.6	8.4	0.021	0.899
2.07	-	0.001	1.02
2.33	4.0	0.010	0.963
2.6	8.0	0.016	0.933
2.9	12.5	0.024	0.855
3.01	15.0	0.028	0.862
3.5	22.9	0.045	0.761
4.1	33.4	0.071	0.609
4.65	43.8	0.113	0.360
5.0	50.0	0.164	0.064

It was later found that buffers at pH 0.5 and pH 5.5 would be useful. The compositions of these were 50 ml 0.1 M citric acid, 25 ml 1.0 M HCl and 25 ml water; and 50 ml 0.1 M citric acid, 11.5 ml 1.0 M NaOH and 38.5 ml water respectively. The ionic strength of these solutions was not calculated accurately but is higher than 0.175, therefore no salt was added.

chloride was added to the buffers to give a constant ionic strength of 0.175 M for most of the buffers. The composition of the buffers is shown in Table 5.

The kinetic parameters are shown in Table 6 and graphs of Vmax and Vmax/Km are plotted in figure 12. The graphs show bell-shaped curves, suggesting the involvement of two important ionisable groups in the enzyme substrate complex and the free enzyme respectively, in the catalytic reaction.

For a bell-shaped pH dependence curve, the catalytically important form has one group protonated, and the other ionised (EH^-). The other forms EH_2 and E^{2-} contribute at the extremes of the pH range. The equation of the bell curve may be represented by the equation

$$\frac{EH^-}{E\ total} = \frac{K_1H}{K_1H + H^2 + K_1K_2}$$

(Alberty & Massey 1954) where H is the hydrogen ion concentration, and K_1 and K_2 are the dissociation constants of the ionisable groups. If K_1 and K_2 are widely separated, then the form EH^- will be the only one significantly present at the pH optimum, (the point mid way between the two pK values for the groups). In this case the values of pK may be estimated as the mid points along the two sides of the curve. However if the two pK values are close together, the other forms of the enzyme are also significantly present and the values of pK are <u>not</u> at the mid point of the sides of the bell, but should be calculated from the equation.

The pK values were calculated from the equation

$$y = \left(\frac{K_1H}{K_1H + H^2 + K_1K_2} \right) x$$

by computation. y is the

TABLE 6

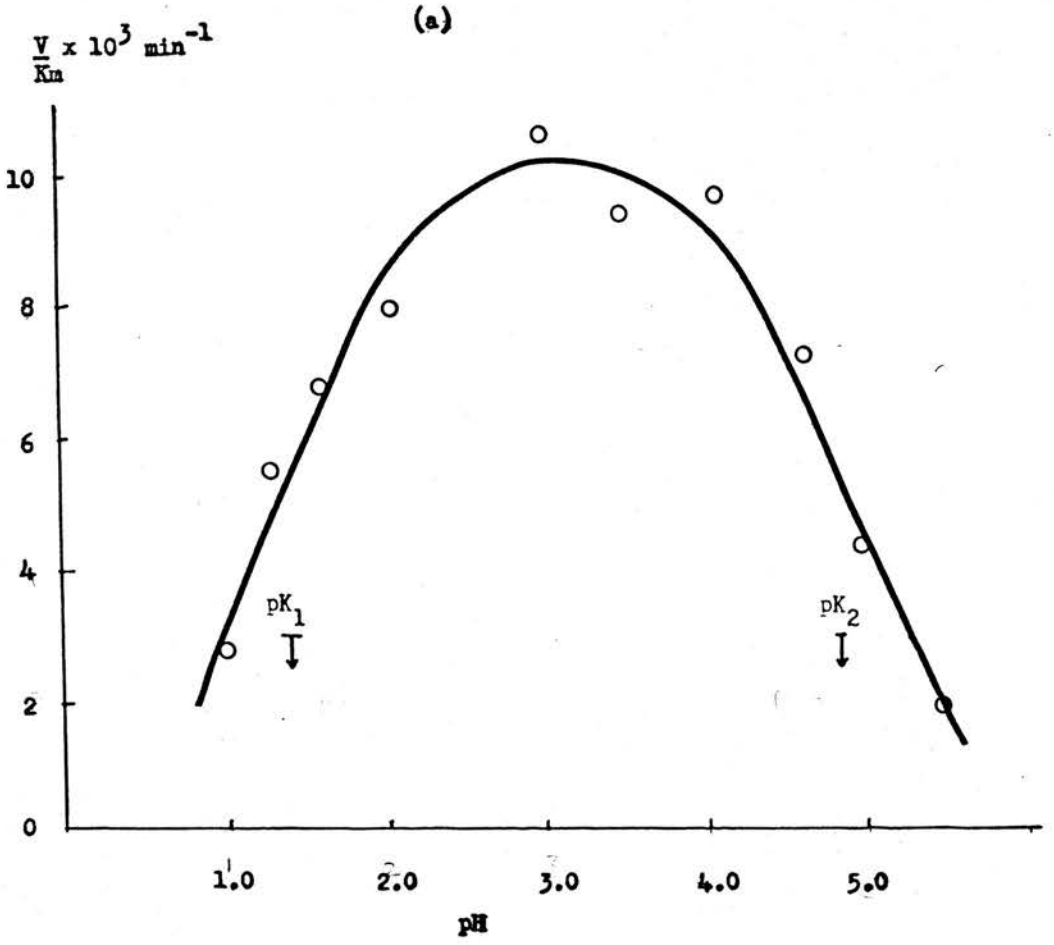
Variation of the kinetic parameters with pH for the hydrolysis of ac-Phe-Leu-Val-His-amide by pepsin C. 0.05 M citrate buffers, 37°.

pH	Vmax $\times 10^4$ (mM min^{-1})	Km (mM)	V/Km $\times 10^4$ (min^{-1})	k cat (s^{-1})
1.0	56.5	1.97	28.4	2.48
1.3	101.0	1.82	55.5	4.43
1.6	112.0	1.64	68.3	4.91
2.07	138.0	1.72	80.0	6.05
3.01	180.0	1.68	107.0	7.89
3.5	170.0	1.8	94.5	7.46
4.1	182.0	1.86	97.0	7.98
4.65	133.0	1.84	72.3	5.83
5.0	82.0	1.89	43.4	3.6
5.5	41.0	2.2	18.6	1.8

Estimates of pK_1, pK_2 and x (see section 6.7)

for V max v. pH, 1.5, 4.9, 185.

for V/Km v. pH, 1.3, 4.9, 105.



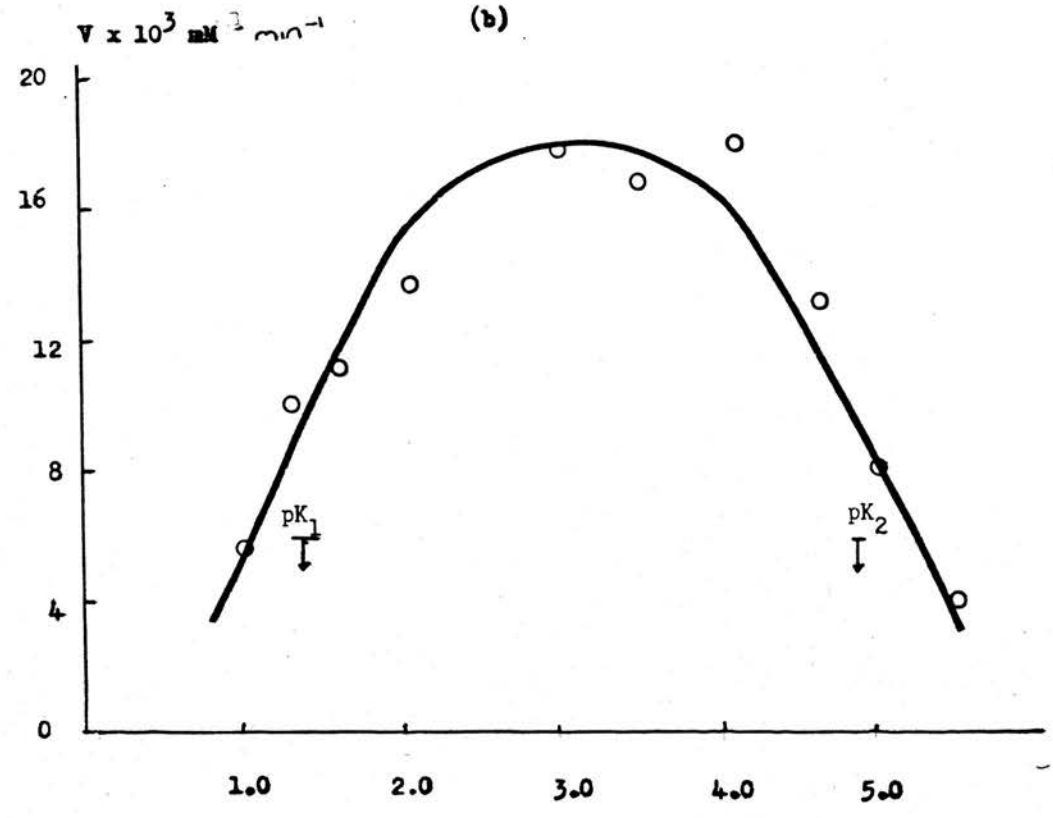


FIGURE 12 pH dependence of (a) $\frac{V}{Km}$ and (b) V max for the hydrolysis of Ac-Phe-Leu-Val-His-amide by pepsin C at 37°C (enzyme concentration 1.4 μg ml^{-1}).

observed experimental value (i.e. V, or V/Km) and x is a scaling factor to bring the size of the bell to the correct fractional level, i.e. $\frac{y}{x} = 1$ if pK_1 and pK_2 are widely separated and only EH^- contributes to the curve, or is less than one if the values of pKa are close. Experimental values of pH and y, together with estimates of pK_1, pK_2 and x were given to the computer. The programme altered the initial estimates by an iterative procedure until the best fit of the experimental points was obtained. The value of x required showed that, in fact the EH^- form was the only one contributing to the activity at the pH optimum (i.e. x = value of y at pH optimum). Calculations were made in terms of hydrogen ion concentration and the results have been converted back to pK values.

pKs of the groups on the free enzyme were found to be 1.42 $\left[\pm 0.06\right]$ and 4.88 $\left[+0.02, -0.03\right]$ and on the enzyme substrate complex 1.37 $\left[+0.09, -0.07\right]$ and 4.87 $\left[+0.03, -0.04\right]$. Thus the pK of the groups on the enzyme do not change significantly on binding substrate.

Attempts to perform an experiment at pH 0.5 were unsuccessful. The buffer in the ninhydrin reagent was not strong enough to cope with the very acid conditions, and when the reagent was modified to account for this, very high blank values were obtained with the substrate. From electrophoretic measurements at pH 3.6 this appeared to be due to the partial hydrolysis of the amide bond. Thus two spots were identified, the major one corresponding to the amide and also one of lower mobility, probably corresponding to free acid. Release of ammonia into the solution would result in the high blank values which were observed.

Since the value of Km for the peptide is independent of pH, but kcat varies with the pH value, the hydrogen ion may be considered as a non-competitive activator up to pH 3.0 and a non-competitive inhibitor between pH 3.0 and pH 5.0. This suggests that pepsin C is an equilibrium enzyme with respect to this substrate, i.e. Km $\simeq$ Ks.

6.8 Hydrolysis of ac-Tyr-Leu-Val-His-methyl ester and the related amide

It has been mentioned (section 6.5a) that these two peptides did not appear to follow classical Michaelis-Menten kinetics. Figures 13 and 14 show the pattern of behaviour in initial velocity studies. The plots of initial velocity against substrate concentration show the usual approach to a plateau region, followed by a second similarly shaped region of the curve. The deviation from classical kinetics is emphasised when the data is transformed for one of the linear plots, for example S/v against S, as shown in the figures. The data appear to fall into two distinct groups, different kinetic parameters being suggested by the points from the low and high substrate concentration ranges respectively. A single data point seems to be intermediate for ac-Tyr-Leu-Val-His-amide. The parameters for the different substrate ranges at pH 2.07 and 37°C were shown in Table 4 (section 6.6). A form of substrate activation appears to be detectable at high substrate concentrations. The possible reasons for this behaviour will be covered in the discussion, but evidence that there is a single substrate present and that only one enzyme is contributing to the hydrolysis is quite good, as discussed elsewhere, and the results are reproducible.

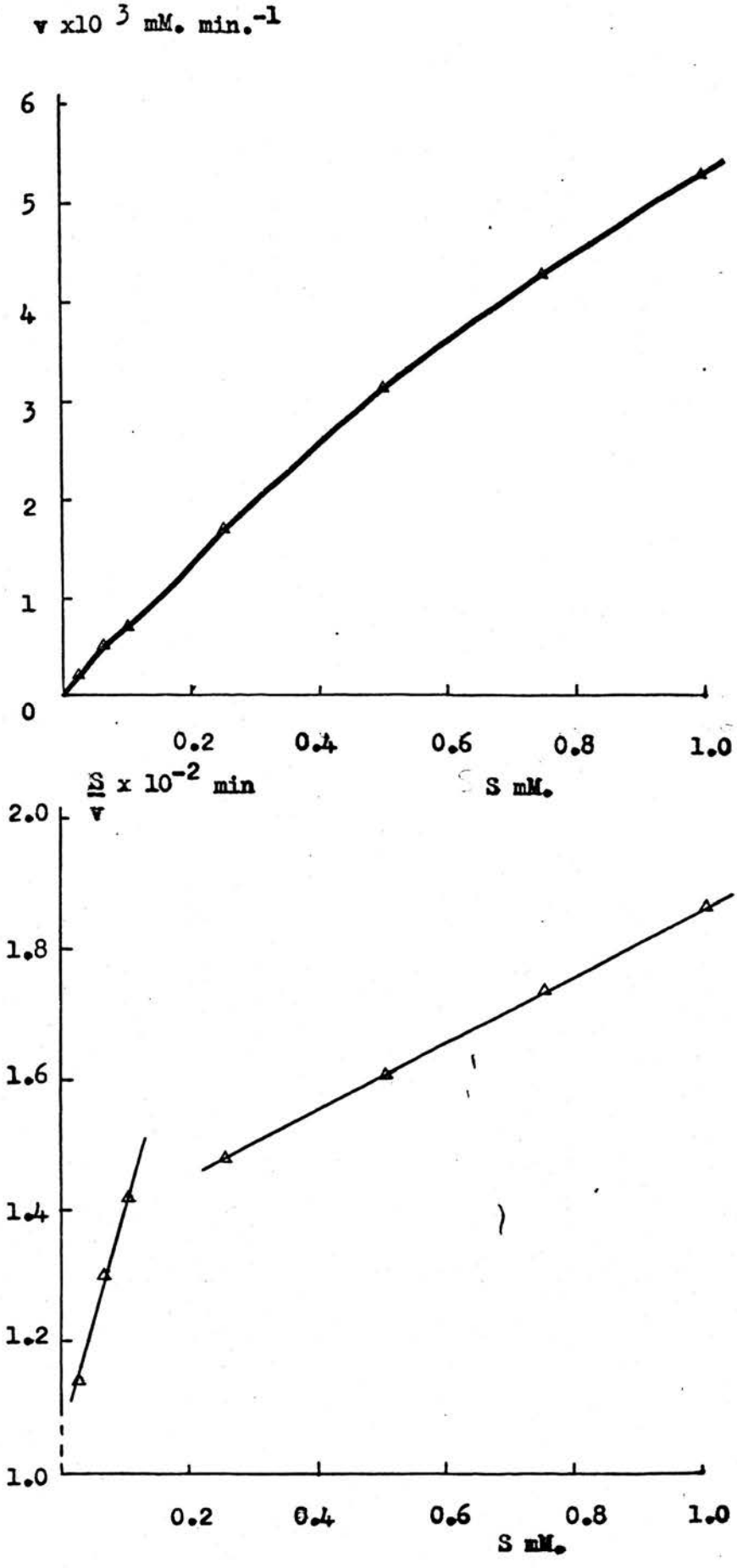


FIGURE 13 Kinetics of the hydrolysis of Ac-Tyr-Leu-Val-His-methyl ester. Enzyme concentration = 1.4 μg ml.$^{-1}$

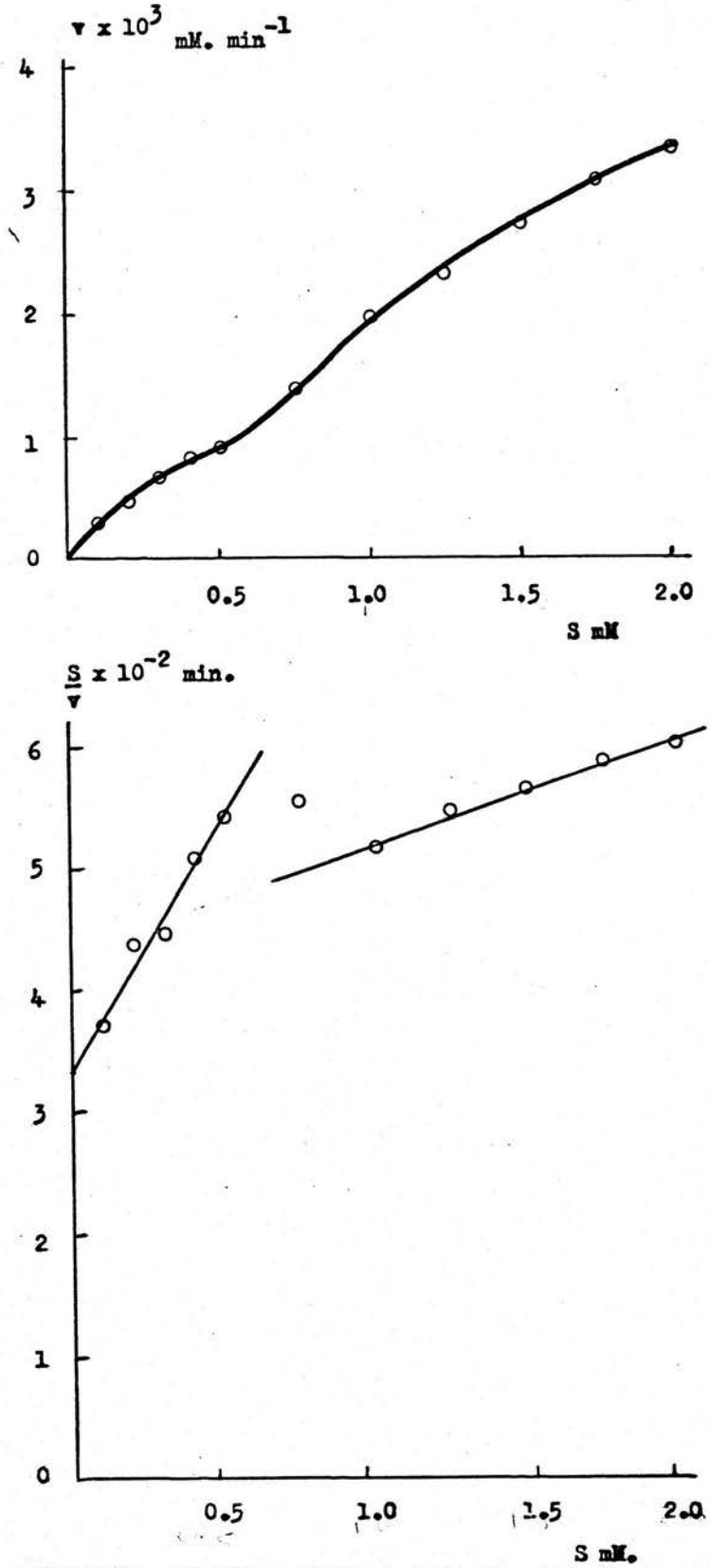


FIGURE 14 Kinetics of the hydrolysis of Ac-Tyr-Leu-Val-His-amide. Enzyme concentration 1.4 µg ml.$^{-1}$

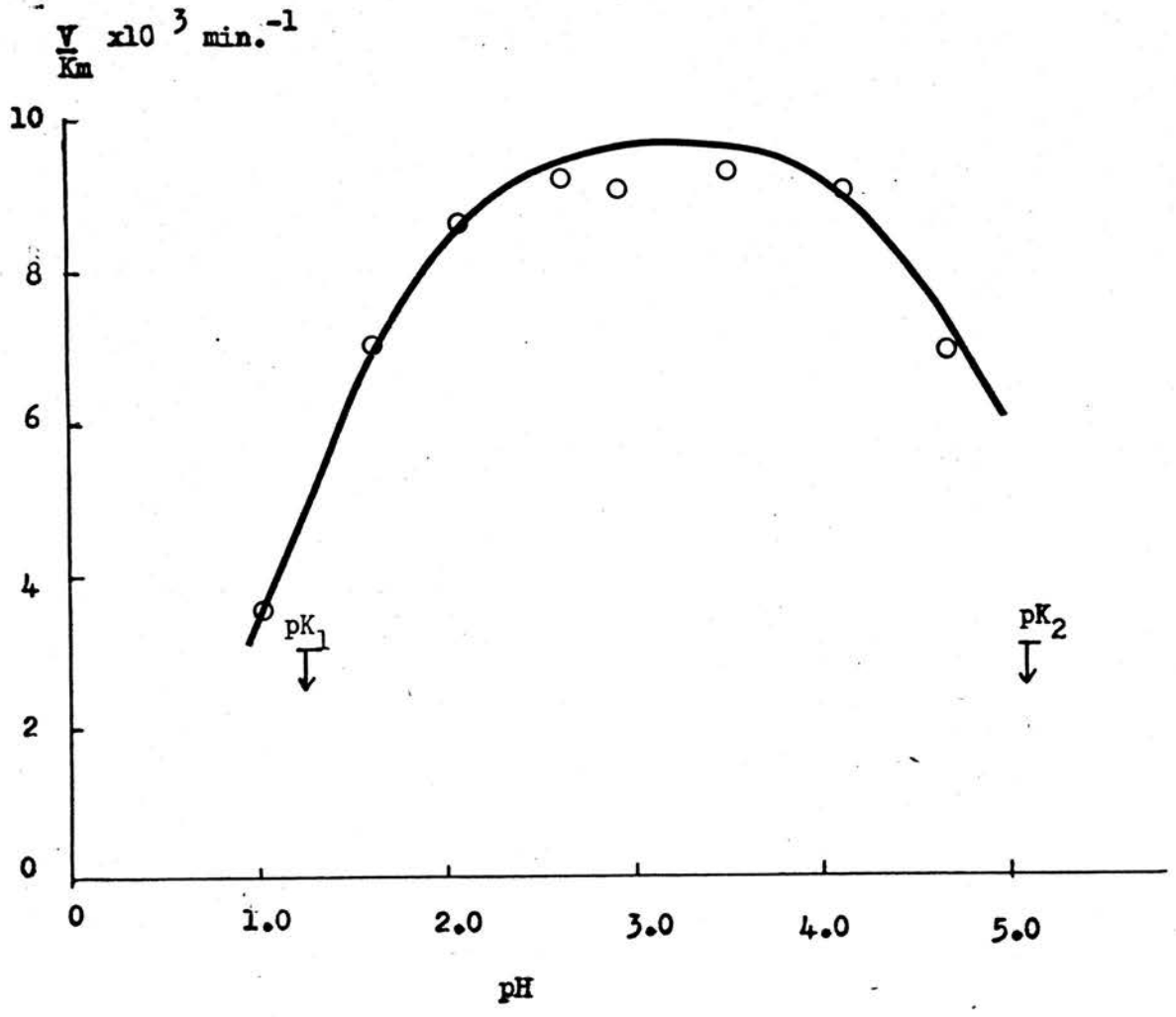


FIGURE 15 pH dependence of $\frac{V}{Km}$ for the peptide Ac-Tyr-Leu-Val-His-methyl ester, measured as the tangent at zero substrate concentration to a plot of v against S.

The hydrolysis of the ester has also been followed over a range of pH values. The activation effect has been observed over the entire range of the experiments and the parameters measured for low substrate concentrations follow a similar bell-shaped curve of dependence upon pH as did those for the peptide ac-Phe-Leu-Val-His-amide. pK values for the dissociation of groups in the enzyme are 1.26 [+0.05, -0.06] and 5.11 [+0.05, -0.03] cf. 1.42 and 4.88 found previously (see figure 15). Since these estimates are based on rather few data, the apparent difference between the pK values found with the two substrates, may not be significant.

Although a detailed study of the parameters obtained by measurements of the rate of hydrolysis at high substrate concentrations was not made at various pH values, the initial velocities at 0.4 mM substrate show a similar pattern. The apparent Km values appear to be unaffected by change in pH over the range studied.

Sufficient data have not been obtained for the peptide ac-Phe-Leu-Val-His-methyl ester to determine the kinetic parameters in the high range of substrate concentration.

6.9 The effect of ac-Ala-Leu-Val-His-methyl ester on the hydrolysis of ac-Tyr-Leu-Val-His-methyl ester

The observation by Wang and Hofmann (1976b) that small non-susceptible peptides could act as activators in the hydrolysis of peptide substrates by pepsin A prompted the following investigation. 0.5 mM ac-Ala-Leu-Val-His-methyl ester, which is not hydrolysed by pepsin C under the conditions used, was added to incubation mixtures

of the tyrosyl peptide in the low concentration range. No effect on the rate of hydrolysis was observed, the peptide apparently not reacting near the active site of the enzyme, either in an activating or inhibiting capacity. This could therefore suggest that it is the aromatic portion of the hydrolysed peptide which is responsible for the activation observed at high concentrations of substrate. Alternatively, it is possible that an activating effect is being exactly countered by an inhibiting one at the particular concentrations used.

6.10 Inhibition of the hydrolysis of ac-Phe-Leu-Val-His-amide with acetyl-L-phenylalanine

The studies were carried out at pH 3.01, where the uninhibited rate was greatest, and where the effect of product inhibition might be most marked.

a) Variation of initial velocity with inhibitor concentration

An initial experiment was performed over a range of inhibitor concentrations (0-30 mM), using 1.0 mM substrate concentration throughout. A Dixon plot of $\frac{1}{v}$ against I allows the estimation of the inhibition constant Ki whether the inhibition is non-competitive or competitive (Dixon & Webb 1964). The type of inhibition cannot be determined by this method, but it is useful for deciding the range of inhibitor concentration to use for studying the variation of the kinetic parameters with inhibitor, leading to the evaluation of the inhibition type. The results of the initial experiment can also be used to standardise subsequent experiments against variation in enzyme activity. Hence comparison of these results at 1.0 mM substrate concentration with values found

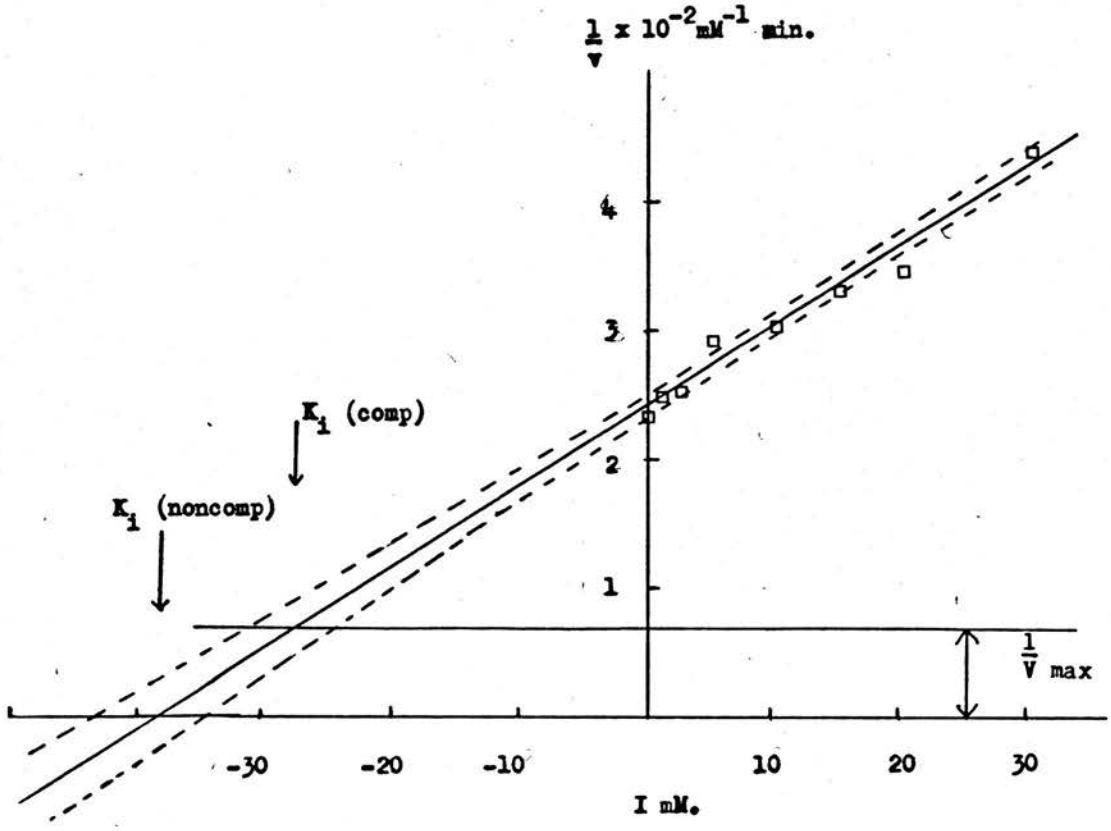


FIGURE 16 Dixon plot for the hydrolysis of Ac-Phe-Leu-Val-His-amide in the presence of Ac-L-Phe at pH3.01 and 37°C. The dotted line represents the error boundary of the linear regression from which errors on K_i may be estimated.

at corresponding concentrations of substrate and inhibitor in the study of the dependence of the kinetic parameters with inhibitor concentration, will show if unexpectedly low activities are found. Corrections may be made accordingly.

Results for the initial experiment are shown in figure 16. The slope is 6.2 $\pm$ 0.52 and the intercept 241 $\pm$ 7.5. For simple non-competitive inhibition, Ki is given by the point of intersection of the linear regression line with the -x axis. If the inhibition is competitive, Ki is the numerical value of x where the regression line and a horizontal line at the value of Vmax for the uninhibited reaction intersect. Errors on the values of Ki were found from the error boundaries of the regression line (shown by dotted lines on the figure) which were calculated by computer. The errors are represented by the points where the boundaries cut the x axis and the $\frac{1}{V\ max}$ line respectively.

Thus for non-competitive inhibition Ki has a value of 38.74 $\left[+4.61, -3.89\right]$ and for competitive inhibition Kiis 27.5 $\left[+3.2, -3.0\right]$.

An alternative method for determining Ki for non-competitive inhibition has been described by Airas (1976) using the same computer programme as that used for the direct linear plot of Eisenthal and Cornish-Bowden (1974). If $\frac{1}{I}$ replaces S in the input and v is entered as usual, the output normally representing Km, is now Ki. Other uses of the programme are also described. An advantage of the method is that transformation of the unknown variable (v) is not required, and thus it is probably statistically more valid than the linear regression method described above. The value obtained by this method was 38.4 in quite close agreement

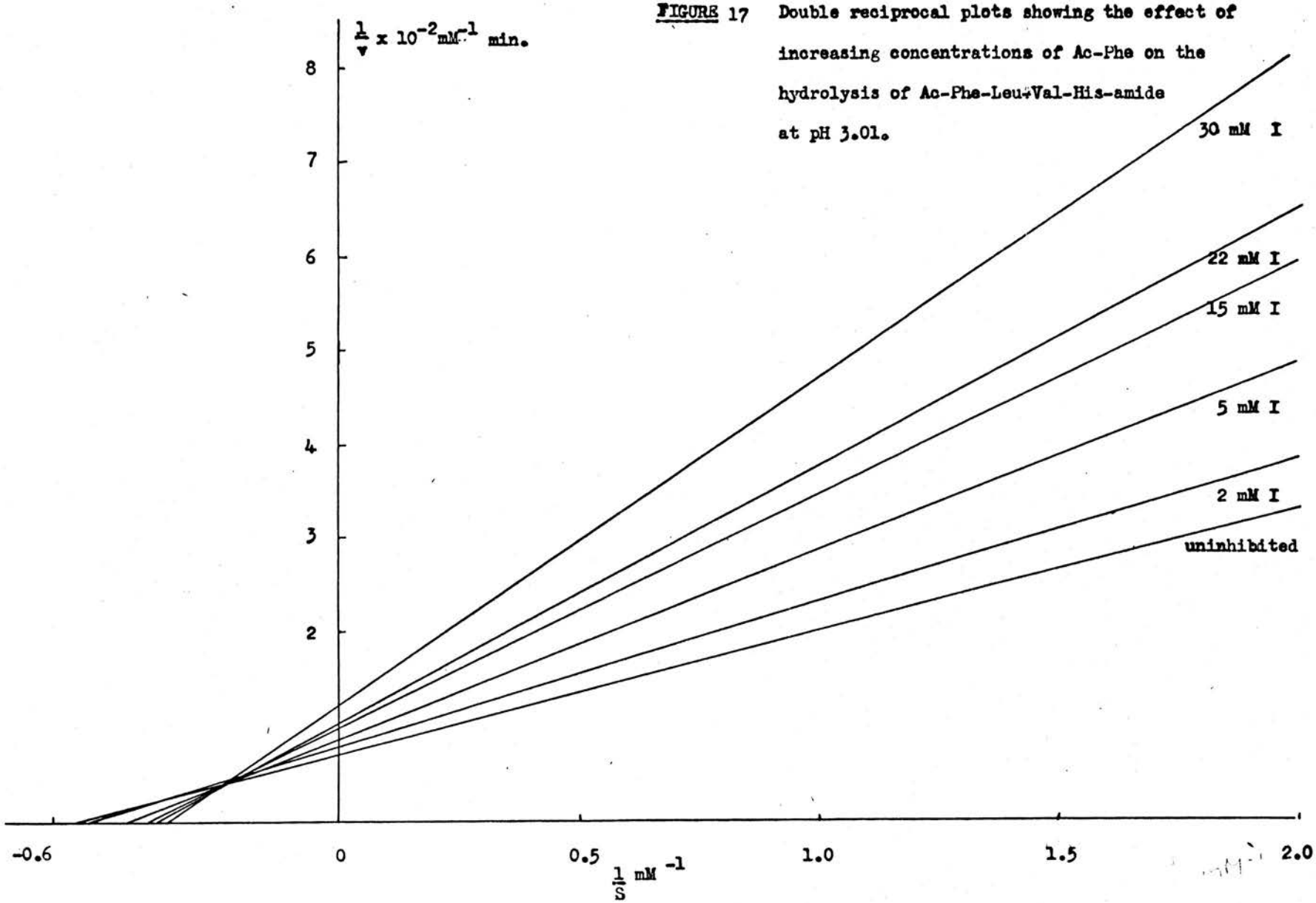


FIGURE 17 Double reciprocal plots showing the effect of increasing concentrations of Ac-Phe on the hydrolysis of Ac-Phe-Leu-Val-His-amide at pH 3.01.

with the method obtained by the Dixon plot.

b) Variation of the kinetic parameters with inhibitor concentration

Experiments were performed at inhibitor concentrations between 0 and 30 mM. A new stock solution of enzyme was used for these studies and it appeared to have a lower activity than that used for the pH dependence studies. The reason for this is not clear, but it is possible that some inactivation had taken place, either through a contaminant being inadvertently introduced, or through storage.

The data from the inhibition studies have been characterised according to the nomenclature of Cleland (1963), that is the pattern of lines formed in a double reciprocal plot. Values of Km and V were calculated by the direct linear plot method rather than by linear regression and the lines constructed on the basis of these results. The data are represented in figure 17, and show a form of non-competitive inhibition sometimes called mixed inhibition, since both Km and Vmax are affected, although the lines intersect (cf uncompetitive inhibition). Replots of the slope, Km/Vmax, and intercept, $\frac{1}{Vmax}$, against inhibitor concentration give straight lines, the horizontal intercepts giving values of Ki_{slope} and $Ki_{intercept}$ respectively.

The replots are shown in figure 18. The slopes were calculated by computation of the linear regression through the data. The graph of slope against inhibitor concentration has a slope 0.67 ± 0.061 and an intercept 14.54 ± 1.01. Ki_{slope} is 21.69 $\left[+3.56, -2.99\right]$. The graph of intercept against inhibitor concen-

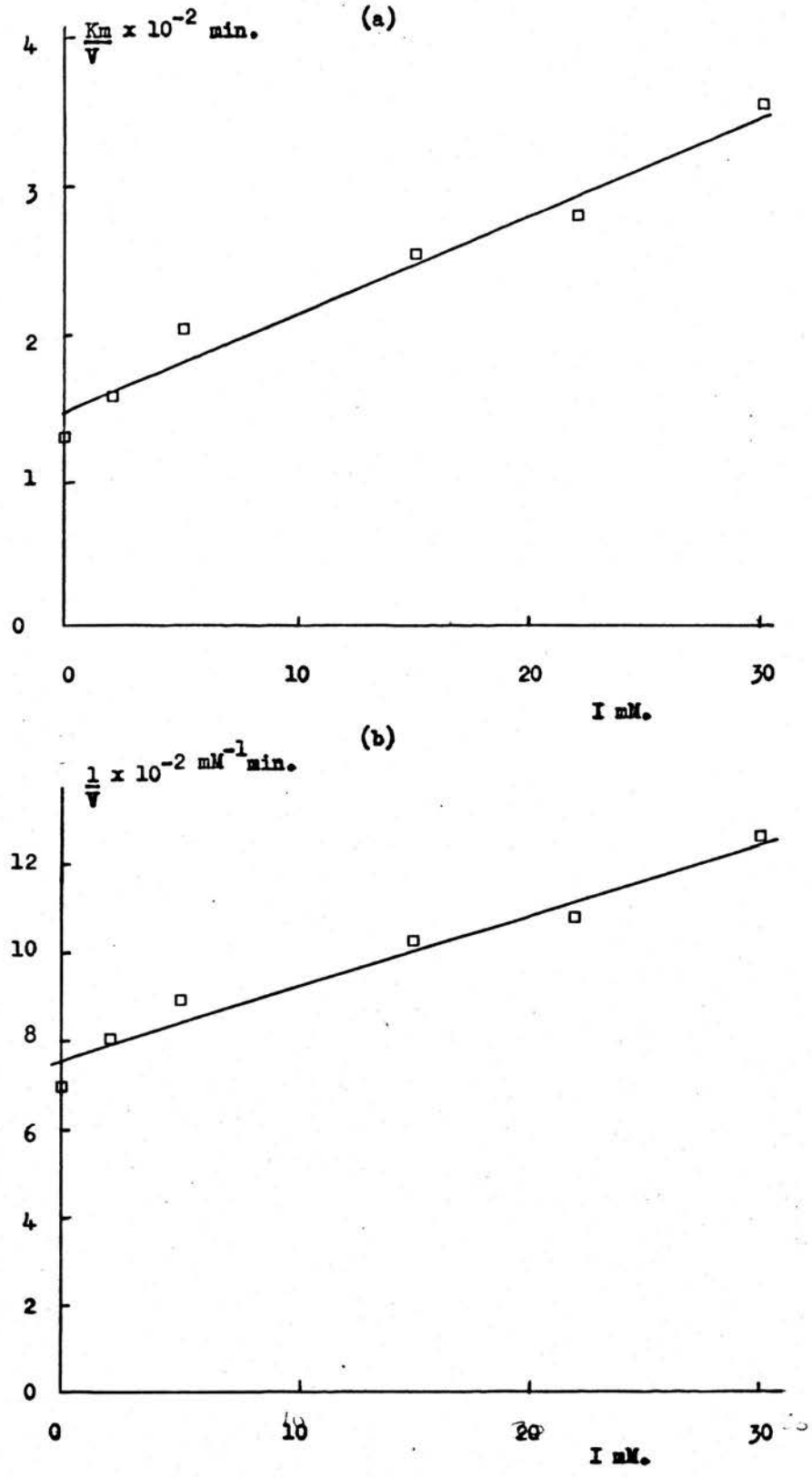


FIGURE 18 Replots of (a) slope and (b) intercept of the double reciprocal plot, against inhibitor concentration, for the hydrolysis of Ac-Phe-Leu-Val-His-amide in the presence of Ac-Phe, at pH 3.01 and 37 C.

tration has a slope of 0.164 ± 0.018 and intercept of 7.58 ± 0.0298. $Ki_{intercept}$ is 46.37 $\left[+7.33, -5.92\right]$. The errors on Ki were calculated from the error boundaries of the regression lines as described above.

Velocities of the 0.5 mM inhibitor concentration have been increased to allow for the low enzyme activity, by comparison with the initial experiment. Since non-competitive inhibition was observed for acetyl phenylalanine at pH 2 for pepsin A (Kitson & Knowles 1971a) but weak competitive inhibition was found at pH 4.3, it was considered that the partial ionisation of the carboxyl group at pH 3.01 might have been responsible for the mixed nature of the inhibition found in these experiments. However, at pH 2.07 and 30 mM inhibitor also showed that both Km and V max were altered.

In accordance with Cleland (1963) non-competitive inhibition by a product molecule is consistent with its being the first product released from the enzyme. Inhibition studies with the other products of hydrolysis, i.e. Leu-Val-His-amide, were not possible owing to the monitoring system which has been used; the peptide would produce too high a blank value, bringing readings outside the sensitive range of the instrument.

SECTION 7. COMPARATIVE STUDIES WITH PEPSINS A and C

7.1 Comparison of the activities of pepsins A and C from pig and man towards peptide substrates

A similar approach to that described in section 6.4 for pepsin C was adopted for the other enzymes, using enzyme concentrations comparable to those which gave a rapid rate of hydrolysis with pig pepsin C. Electrophoresis of prolonged incubation mixtures showed that susceptible peptides were hydrolysed at the same unique site as when hydrolysed by pepsin C. Neither Ac-Ala-Leu-Val-His-methyl ester nor the corresponding amide were hydrolysed by the other enzymes. Ac-Phe-Leu-Val-His-methyl ester, the corresponding amide and Ac-Ala-Phe-Leu-Val-His amide were hydrolysed by all the enzymes and further studies were made with the two amides. The three tyrosine containing peptides ac-Tyr-Leu-Val-His- as the free acid, the methyl ester and the amide were all digested by gastricsin (human pepsin C) but digestion by the pepsins A was not observed under the conditions used initially.

a) Peptic hydrolysis of phenylalanine containing peptides

1. Amides

The parameters Km and kcat were estimated as previously described (section 6.3) at pH 2.07 and are presented in Table 7. Because of the low solubility of ac-Ala-Phe-Leu-Val-His-amide, sufficient solid was weighed to make a solution that was nominally 0. 5 mM to use in all the experiments with the enzymes. The solution was treated as described in section 6.6b, and it was found that

TABLE 7

Peptic hydrolysis of ac-Phe-Leu-Val-His-amide and ac-Ala-Leu-Val-His-amide at pH 2.07, 37°

ac-Phe-Leu-Val-His-amide

	Pepsin C (pig)	Pepsin A (pig)	Gastricsin (human)	Pepsin (human)
Km (mM)	1.72	1.5	2.95	3.0
k cat (s^{-1})	6.05	0.35	0.6	0.27
k cat/Km($s^{-1}mM^{-1}$)	3.52	0.24	0.20	0.09

ac-Ala-Phe-Leu-Val-His-amide

	Pepsin C (pig)	Pepsin A (pig)	Gastricsin (human)	Pepsin (human)
Km (mM)	0.72	0.85	0.59	0.84
k cat (s^{-1})	7.54	5.47	0.33	3.38
k cat/Km($s^{-1}mM^{-1}$)	10.42	6.46	0.56	4.02

73% of the solid had dissolved . The values obtained for pepsin C agree with those obtained previously (section 6.6, Table 4). It can be seen that at pH 2.07, pepsin C digests ac-Phe-Leu-Val-His amide very much more rapidly than do the other enzymes. Pepsin C is about 10 times as active as porcine pepsin, but gastricsin is only $2\frac{1}{2}$ times as active as human pepsin. The higher Km values for the human enzymes are interesting to note.

The additional alanyl residue in the second peptide has a marked effect on both pepsins A, increasing the measured activity (kcat) by more than tenfold, while the activity of pepsin C is only slightly increased, and for gastricsin is decreased. The general lowering of the Km values, implying a tighter binding of the substrate to the enzymes, leads to an increase in "efficiency" as measured by kcat/Km, compared with ac-Phe-Leu-Val-His-amide. This is about thirtyfold for the pepsins A but only threefold for the pepsins C.

2. Ester

A detailed study of the hydrolysis of ac-Phe-Leu-Val-His-methyl ester has not been made, because of the complex effects which were observed with pepsin C. However the initial velocities have been measured for the hydrolysis of a 0.2 mM solution at pH 2.07. The velocities measured as μmole product per mg enzyme per minute (μmole mg^{-1} min) were found to be 0.738 for pepsin C, 0.074 for gastricsin, 0.037 for porcine pepsin A and 0.04 for human pepsin. The pattern observed is thus similar to that seen in the hydrolysis of the corresponding amide.

TABLE 8

Comparison of the hydrolysis of some peptides by

Pepsin C and Gastricsin

Substrate	pH 37°	So (mM)	Pepsin C k cat/Km (s^{-1} mM^{-1})	Gastricsin k cat/Km (s^{-1} mM^{-1})
ac-Tyr-Leu-Val-His-OMe	2.07	0.025 - 0.1	3.77	1.03
	3.01	0.025 - 0.1	4.21*	1.26
ac-Tyr-Leu-Val-His-NH_2	2.07	0.1 - 0.5	1.34	0.28

* value calculated from pH dependence studies (section 6.8)

Hydrolysis of ac-Tyr-Leu-Val-His-methyl ester and the corresponding amide by pepsin C and gastricsin.

At pH 2.07, the rate of hydrolysis of both ester and amide by gastricsin was directly proportional to the substrate concentration up to 0.1 mM substrate. Higher substrate concentrations seemed to yield an activation similar to that described for pepsin C. The kcat/Km values obtained from the gradient of these lines are compared with those for pepsin C, measured in the low concentration range in Table 8. Individual estimates for the parameters were obtained for the ester at pH 3.01 with gastricsin, kcat is $0.22s^{-1}$ and Km 0.17 mM. Pepsin C is about three times as active as gastricsin towards these substrates at pH 2.07.

SECTION 8. DIFFERENTIAL ASSAY FOR MEASURING HUMAN PEPSIN AND GASTRICSIN IN A MIXTURE

8.1 Introduction

The ability of gastricsin but not human pepsin to hydrolyse ac-Tyr-Leu-Val-His-amide, at the enzyme concentrations tested, together with the apparent stability of the peptide, suggested that it might be used in setting up a differential assay to measure the relative levels of the two enzymes in a mixture. This peptide would allow the assay of gastricsin, and acetyl phenylalanyldiiodotyrosine would provide a suitable substrate for assay of the pepsin. Ideally, activity towards the other peptide would be zero for each enzyme, but cross reactivity can be allowed for by formulating simultaneous equations which take into account the activity of each enzyme towards each substrate.

A calibration curve for the hydrolysis of ac-Tyr-Leu-Val-His-amide by pure gastricsin, and one for the hydrolysis of ac-Phe-(I_2)-Tyr by pepsin A were constructed. A series of mixtures of the enzymes, of known composition were used in both assays, and from these data, the contribution of the enzyme with the minor activity was determined. Separate calibration curves were not drawn up to determine the cross reactivity, both to preserve substrate, and as the levels of absorbance above the enzyme blank would be very low, and hence difficult to measure accurately.

8.2 Assay for gastricsin with ac-Tyr-Leu-Val-His-amide

It was decided to perform the assay at pH 3.01 since the initial

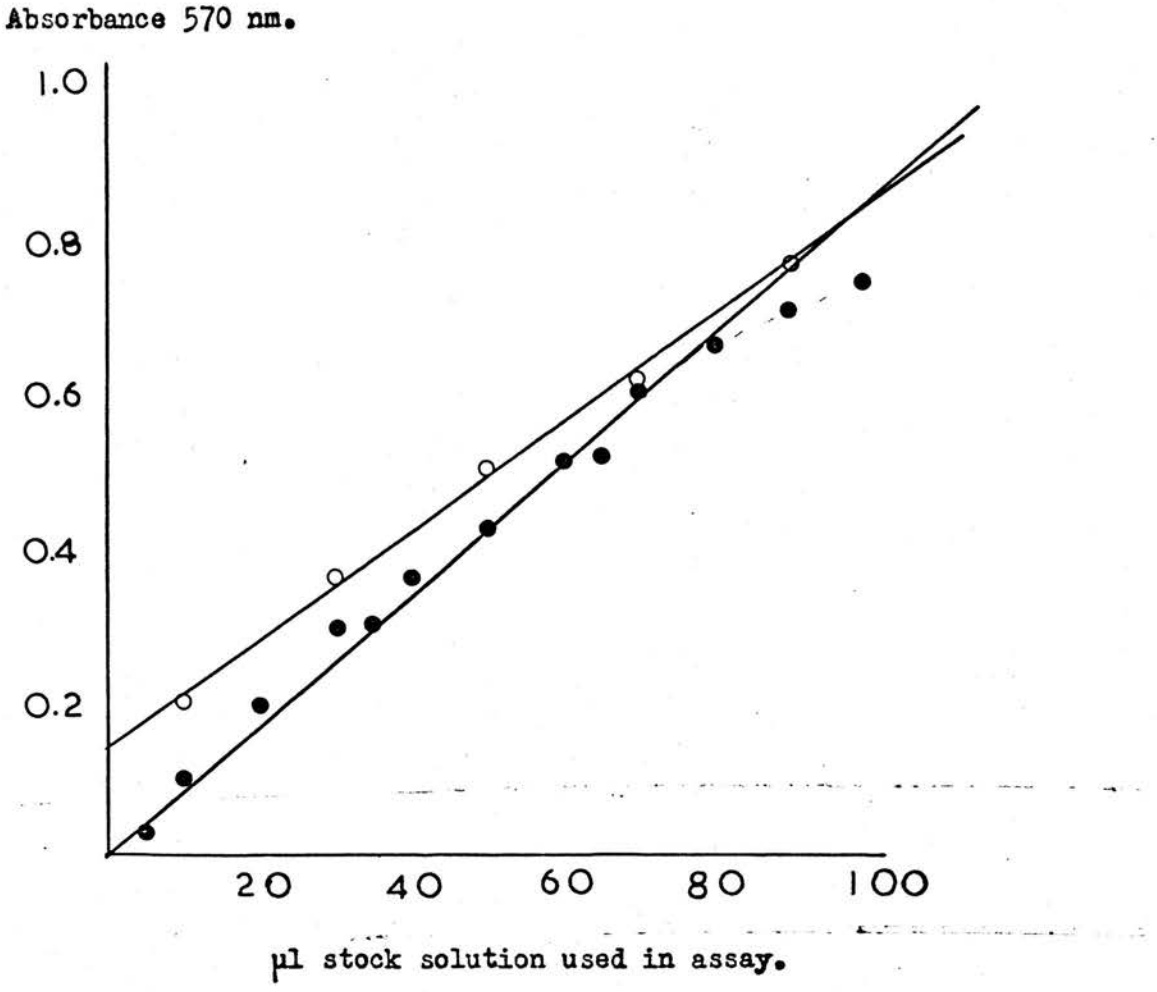


FIGURE 19 Calibration curve for the hydrolysis of Ac-Tyr-Leu-Val-His-amide by gastricsin (G) • , and a mixture of gastricsin and human pepsin (P) o ,such that G+P = 100 µl
Stock solutions : P = 1.0 mg ml^{-1} G = 0.4 mg ml^{-1}.
Incubations were performed at pH 3.01 and 37°C
NB. Points above 80 µl were not included in the regression for hydrolysis by gastricsin alone (•)owing to the apparent curvature beyond this point.

acYLVH

velocity at this pH was found to be 50% more than the rate at pH 2.07. This experiment was also used as a guide for the reaction conditions which might be tested.

Thus, 0.5 ml of enzyme in water (~ 25 μg) was equilibrated to 37°C and then 0.5 ml of a 1.0 mM solution of the substrate in 0.05 M citrate buffer pH 3.01 (also at 37°C) was added. After thorough mixing and incubating for 20 minutes the reaction was stopped by the addition of 1 ml of ninhydrin reagent and the colour was developed as described in the assay with Ac-Phe-(I_2)Tyr, (section 2.2). Blank reactions for enzyme and substrate were also performed, and the Δabsorbance at 570 nm due to the peptide hydrolysis was 0.425. These conditions were thus suitable for constructing the calibration curve.

Production of the calibration curve

Assays were performed in duplicate as described above. Enzyme blanks were performed, also in duplicate, where 0.5 ml of enzyme in water and 0.5 ml 0.05 M citrate buffer were treated with ninhydrin. A duplicate substrate blank and a buffer blank were performed rather than using blanks with denatured enzyme and substrate, to avoid waste of the peptide. The contribution of colour due to substrate only was subtracted from all the assays. The enzyme blank values were plotted against enzyme concentration, and a line constructed by least squares analysis to give an accurate value of the blank contribution at each enzyme concentration. The calibration curve is shown in figure 19. It is linear up to a level of about 35 μg enzyme (representing 40% hydrolysis). The best fit line was calculated constraining it to pass through the origin (and

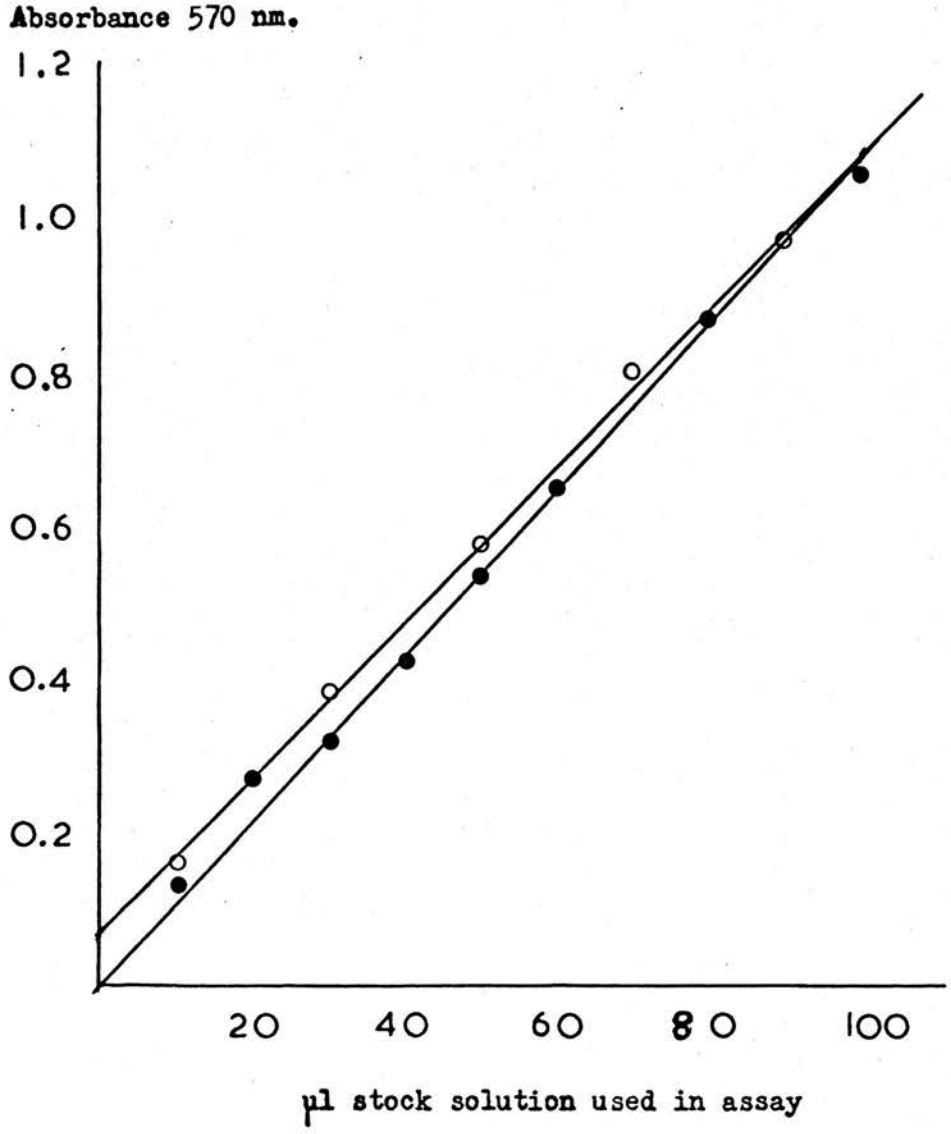


FIGURE 20 Calibration curve for the hydrolysis of Ac-Phe-(I_2)-Tyr by human pepsin (P) ●, and a mixture of human pepsin and gastricsin (G) o ; such that P+G =100 µl.
Stock solutions: P = 1.0 mg ml^{-1} ; G = 0.4 mg ml^{-1}.

excluding the points where curvature was observed). The slope was $0.85 \times 10^{-2} \pm 1.8 \times 10^{-4}$.

8.3 Assay for pepsin using ac-Phe-(I_2)Tyr (APD)

The conditions for the assay were those of Tang (section 2.2b). The incubation mixture was modified to allow the use of 100 µl of enzyme in water (for slight greater flexibility in choice of sample volume) and 0.0269 M HCl was employed to give the same final pH. The calibration curve is shown in figure 20. When constraining the line to pass through the origin, the slope is $1.074 \times 10^{-2} \pm 1.6 \times 10^{-4}$.

8.4 Assays using a mixture of enzymes

The assays were performed as described above, using enzyme solution containing known quantities of each enzyme. Lines were again computed as shown in the figures. For the acetyl tyrosyl peptide, the gradient is now 0.699×10^{-2} ($\pm 2.23 \times 10^{-4}$) giving an intercept of 0.144 ($\pm$ 0.0128). For the APD assay the gradient is now 1.02×10^{-2} ($\pm 2.37 \times 10^{-4}$) with an intercept of 0.0684 ($\pm$ 0.0136).

From these data, equations can be formulated representing the amounts of enzymes in the mixture. In the experiments described above, the quantity of each enzyme was represented by the number of microlitres of a stock solution. This was later converted to the microgram quantities of the enzymes gastricsin (G) and pepsin (P) present. Hence the equation

$$\Delta \text{ OD (ac-Tyr-peptide)} = 0.0214\ G + 0.00144\ P$$

$$\Delta \text{ OD (ac-Phe-}(I_2)\text{Tyr)} = 0.0102\ P + 0.0017\ G$$

may be solved.

The specific activity found for pepsin with ac-Phe-(I_2)Tyr as substrate, i.e. 0.0102 OD units produced per microgram of pepsin is quite similar to that reported by Tang (1970), who quotes a value of 0.011. He however found that gastricsin did not have any activity towards this substrate. Evidence for the purity of the gastricsin used in this work was discussed in section 5.4b, (i.e. behaviour of the enzyme in polyacrylamide gel electrophoresis). It is possible that gastricsin has an intrinsic activity towards the peptide. Further comparison with Tang's differential assay which uses the assay with haemoglobin to measure total peptic activity, and the assay with the synthetic peptide to find the contribution by pepsin A, have not been made.

The data presented above suggest that the assay system using two synthetic peptides is quite reliable, and it is also easier to carry out, a single general procedure being involved. It has the potential of automation.

SECTION 9. DISCUSSION

The studies on the activity of porcine pepsin C with synthetic peptides have shown that, like pepsin A, it can cause hydrolysis of certain susceptible bonds, despite the finding (Ryle & Porter 1959) that acetyl phenylalanyl diiodotyrosine is resistant to attack by pepsin C. It has been found that for peptides of the type A-Leu-Val-His-B, the rate of hydrolysis increases through the series where A represents acetyl alanyl, acetyl tyrosyl, acetyl phenylalanyl, acetyl alanylphenylalanyl. Methyl ester groups are apparently preferred to amides or the free acid group at B, although the evidence of this is not very substantial. Although the phenylalanyl peptides are hydrolysed more rapidly than the tyrosyl ones, kcat values of about 1.0 s^{-1} for the latter show that these are quite good substrates. This result is as may be expected, considering the pattern of hydrolysis of the B-chain of oxidised insulin, and shows a contrast between the activities of pepsin C and pepsin A. The tyrosyl peptides were only very slowly hydrolysed by pepsin A, in agreement with work published by Fruton (reviewed 1970, 1976) for similar peptides in which a tyrosine residue contributes the carbonyl group to the peptide bond. Therefore some difference must occur between the active sites regions of the two pepsins, such that pepsin C can accommodate the tyrosyl hydroxyl group, but pepsin A cannot. This could perhaps be due to a difference in size of residues in the binding site, or the presence of a group in pepsin C, but not pepsin A, allowing a stabilising hydrogen bond to be formed between the enzyme and the tyrosyl hydroxyl group. A residue with a large side chain at the β carbon might sterically hinder the approach of a tyrosine containing peptide to the correct position

in the active site of pepsin A.

The values of Km which have been measured for the peptides with pepsin C do not show very great variation, with the exception of ac-Tyr-Leu-Val-His ester, when the parameter is estimated from initial velocities measured at low substrate concentrations. The extension of the Ac-Phe-Leu-Val-His-amide with a single alanyl residue at the amino terminus causes about 50% decrease in Km. This may represent tighter binding of the substrate to the enzyme via a secondary locus, in a manner similar to that proposed for pepsin A.

Secondary binding sites may be important when pepsins act on whole protein substrates, when steric hindrance from other parts of the molecule may prevent the residues contributing to the susceptible bond from being tightly bound to the enzyme themselves, but the bond could be held in the right configuration at the catalytic site through secondary enzyme substrate interactions. As the proposed secondary sites are thought to be hydrophobic in nature (Fruton 1976 for a review), this could put constraints on the binding of highly chargedprotein molecules. It should be noted however that the additional alanyl residue in the extended peptide has a greater effect on the rate of hydrolysis by pepsin A from both pig and man than on that by pepsin C, (more than a tenfold increase over the acetyl phenylalanyl peptide , compared with an increase of only fifty percent for pepsin C). For gastricsin, kcat is lower for the extended peptide, although it usually shows a similar pattern of activity to pepsin C. The values of Km for the extended peptide are quite similar for all the enzymes, although for

ac-Phe-Leu-Val-His-amide, Km is apparently twice as great for the human enzymes compared with the porcine ones. The combined effect of changes in both kcat and Km results in a threefold increase in the $\frac{kcat}{Km}$ ratio or "efficiency" for both pepsin C and gastricsin and a thirtyfold one for pepsin A from both pig and man. More data would be required to substantiate the values obtained from these experiments, as the results are for a single estimation of the parameters. The activities agree with single estimations of the initial velocity at a particular substrate concentration performed on a different day. As was mentioned in the results section, the extended peptide was very insoluble, the absolute concentration of the stock solution being measured after centrifuging to remove insoluble material. This made the peptide rather difficult to use and since the values of Km are much higher than the maximum solubility (0.375 mM) their reliability is not certain.

From the comparative studies between the enzymes, pepsin C has been found to be more active than the other enzymes towards the synthetic substrates. This may not be surprising since the initial aim was to design small substrates which pepsin C could hydrolyse. Gastricsin which is the equivalent to pepsin C in man, will also hydrolyse most of the peptides which are attacked by pepsin C. However the activity of gastricsin is very much lower than that of pepsin C towards these small peptides, although they have a similar activity in the haemoglobin assay when measured by the methods of both Ryle and Porter, and Tang (section 2.1).

Despite the hydrolysis in the B chain of oxidised insulin of the alanyl-leucyl bond (residues 14 and 15), in the sequence

-Glu-Ala-Leu-Tyr-Leu-Val-Cys(O_3H)- only pepsin C would hydrolyse the alanyl-leucyl bond in the synthetic peptides at the concentrations tested. The rate was so slow that estimations of the parameters could not be made. It is possible that in the long polypeptide of insulin, other residues are assisting in the binding of the molecule to the enzyme, such that the bond is near the catalytic site, once again implying the importance of secondary binding sites.

In general, the porcine enzymes seem to show relatively greater activity in the hydrolysis of small peptides than do the human enzymes. It is not possible to say whether there is any biological significance in this observation.

The studies of pH dependence of pepsin C show a similar pattern to those described for pepsin A by Denburg et al. (1968), Cornish-Bowden and Knowles (1969), Clement et al. (1968) and others, as was described in the introduction. The pKa values of important groups in pepsin C are about 1.4 and 4.9, higher than those found for pepsin. The data also suggest that no change is found in the pK values of the groups during the formation of the enzyme substrate complex. The pH optimum for hydrolysis of the small peptides by pepsin C is also higher than that for pepsin A.

The study of pH dependence of the parameters on the hydrolysis of ac-Tyr-Leu-Val-His-methyl ester at low concentrations of substrate show slightly different estimations of the pK values of groups on the free enzyme (1.3 and 5.1 respectively) but the lack of data in this study may account for this. Also, as will be discussed later, this peptide did not give classical kinetic observations and this could also be a cause of the discrepancy.

Gastricsin also has a higher activity at pH 3 than at pH 2. The reason why parameters could not be estimated for the tyrosyl peptides with this enzyme at pH 2, but were attained successfully for the phenylalanyl peptides, is not clear. This may be an effect of the tyrosine residue itself as has been suggested for pepsin A. A group on the enzyme might be protonated at pH 2 but ionised at pH 3, allowing better binding of the substrate.

The pK values of groups at the active site of pepsin C are suggestive of carboxylate groups. The work of Kay and Ryle (1971) using covalent labelling of one catalytic residue shows that one of the carboxyl groups in pepsin C is contributed by an aspartyl residue in the sequence -Ile-Val-Asp-Thr-, which is identical to that found for pepsin A in the active site region.

The inhibition experiments at pH 3.01, which by Cleland's nomenclature (1963) show non-competitive inhibition, may be consistent with ordered release of products from the active site. However unlike in the work done with pepsin A at pH 2, the inhibition was not found to be 'simple' linear non-competitive (i.e. the intersection point was not on the $-\frac{1}{S}$ axis in a double reciprocal plot), both Km and Vmax being affected. It was considered that the difference may have been due to the pH used in the present study. At pH 2, the carboxyl group on the inhibitor would be totally protonated, whilst at pH 4.0, competitive inhibition has been noted with some substrates (e.g. Inouye & Fruton 1968; Clement et al. 1968) when the group would be ionised. Thus performing the experiment at an intermediate pH could cause confusion of the results. Repeating the experiment at pH 2.0 with 30 mM inhibitor, showed a similar pattern to that obtained

at pH 3.01 (section 6.10b).

Inhibition with the second product might be expected to show competitive inhibition effects, but this experiment could not be performed using the techniques which have been described. Therefore the evidence is not sufficient to state that there is ordered release of products, or to imply an amino intermediate is involved in the enzyme mechanism which has been suggested for pepsin A, by some workers (see introduction). It is repeated that work of Kitson and Knowles (1971a), which showed non-competitive inhibition by the acyl product, was not consistent with ordered release of products when taken in the context of other studies (Silver & Stoddard 1975). Transpeptidation experiments reported by Ryle (1960a) suggest that an amino enzyme might be intermediate in the reaction of pepsin C with some substrates.

It seems likely that pepsin C will, like pepsin A, show a complex pattern of possible mechanisms of action.

The non-classical kinetics found for the hydrolysis of the tyrosyl peptides, ac-Tyr-Leu-Val-His-methyl ester, and its corresponding amide, prompted further studies to try to examine the relationship between initial velocity and substrate concentration more fully. Experiments were carried out over a wide range of substrate concentrations several times, to establish that the effect was reproducible. The effect has also been observed over a wide range of pH for the peptide ester. The pH dependence curve of $\frac{kcat}{Km}$, measured as a tangent to the v versus S graph at zero substrate concentration, has already been discussed, but it is also worth noting that a similar bell-shaped dependence curve was found

for the initial velocity at a single concentration of substrate contributing to the higher Km, Vmax estimations.

Several causes for the non-linearity of data when a graph of one of the common linear transformations is plotted, have been suggested (e.g. Wharton et al. 1974).

If the enzyme is impure, the substrate may be acted on in non-identical ways, but although it is known that the pepsin C preparation had some contamination with pepsin A (section 5.5) the latter enzyme is inactive in the hydrolysis of the peptide, as shown by independent experiments. An impurity in the substrate preparation could be responsible if it behaved as an activator, but as a similar activation effect has been observed with more than one substrate, this is not very likely. The plot of initial velocity against substrate concentration is more complex than could be explained by two substrates or two enzymes, if each reaction is individually following Michaelis-Menten kinetics, since these would give a composite curve without the intermediary plateau regions. The apparently normal behaviour with other peptides suggest that the complexities do not arise from the enzyme having more than one active site per molecule. The conditions used for the different substrates were similar, so aggregation leading to cooperative effects of enzyme molecules is unlikely to have occurred for some peptides but not others. It is possible that the effect of an intermediary plateau region might have been observed for other peptide hydrolyses if the substrate range had been further extended. The two tyrosyl peptides themselves show different properties of the curve, and thus it may be that for the other peptides only the first portion of a complex curve has been observed.

Teipel and Koshland (1969) have examined curves of the type obtained in the experiments with the tyrosyl peptides. The curve has two points of inflection, with an intermediary plateau region, and the authors conclude that this can only occur if there are more than two substrate binding sites on the enzyme or enzymes present. The saturation of the enzyme follows a complex series of interactions, where cooperative interactions perhaps via conformational changes may be involved. The occurrence of a plateau region in an initial velocity versus substrate concentration curve has also been studied by Bardsley and Childs (1975). They suggest that the mechanism of an enzyme showing this type of kinetics involves three molecules of substrate, for the formation of product. It is evident however that the absolute values of the rate constants, or at least their relationship to each other, determine whether or not an intermediary plateau region will be seen, even when this mechanism is followed. Thus a similar, complex mechanism could outwardly give the appearance of following simple saturation kinetics which were described for most of the substrates.

On the basis of the experimental data found for the peptides ac-Tyr-Leu-Val-His-methyl ester or amide and the literature cited above, a tentative proposal can be made for the mechanism of pepsin C towards these peptides.

At low substrate concentrations it is suggested that classical Michaelis-Menten kinetics are followed, but that as the substrate concentration increases, two further substrate molecules begin to saturate the enzyme molecule at secondary sites. An ES_3 complex is thus formed which causes the more rapid hydrolysis of

the substrate molecule which is at the active site. The mechanism for this process could be very complex, even assuming that the first substrate molecule always bound at the catalytic site. The other substrate molecules may bind cooperatively, but perhaps either one could be bound first. An intermediate ES_2 complex might also break down at a different catalytic rate to form product, substrate and free enzyme, perhaps in more than one step.

As only a few experimental points have been obtained, it has been felt most suitable to apply a highly simplified mechanism to represent the hydrolysis to which to fit the data. Thus the two activating substrate molecules have been considered to bind to the enzyme in a single step. The hydrolysis is considered to be essentially irreversible, and the two activating molecules are assumed to become dissociated after the hydrolysis step. Thus

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$ES + 2S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_3 \xrightarrow{k_4} E + P + 2S$$

Hence $$v = \frac{V_1 S}{K_1 + S + \frac{S^2}{K_3} + \frac{S^3}{K_2}} + \frac{V_2 S}{\frac{K_2}{S^2}\left[K_1 + S + \frac{S^2}{K_3} + \frac{S^3}{K_2}\right]}$$

where $V_1 = k_2 Et$, $V_2 = k_4 Et$

$$K_1 = \frac{k_{-1} + k_2}{k_1}, \quad K_2 = \frac{k_{-3} + k_4}{k_3}, \quad K_3 = \frac{k_1}{k_3}$$

The absolute values of the constants determine whether a marked plateau region occurs. It appears that V_2 must be very much greater

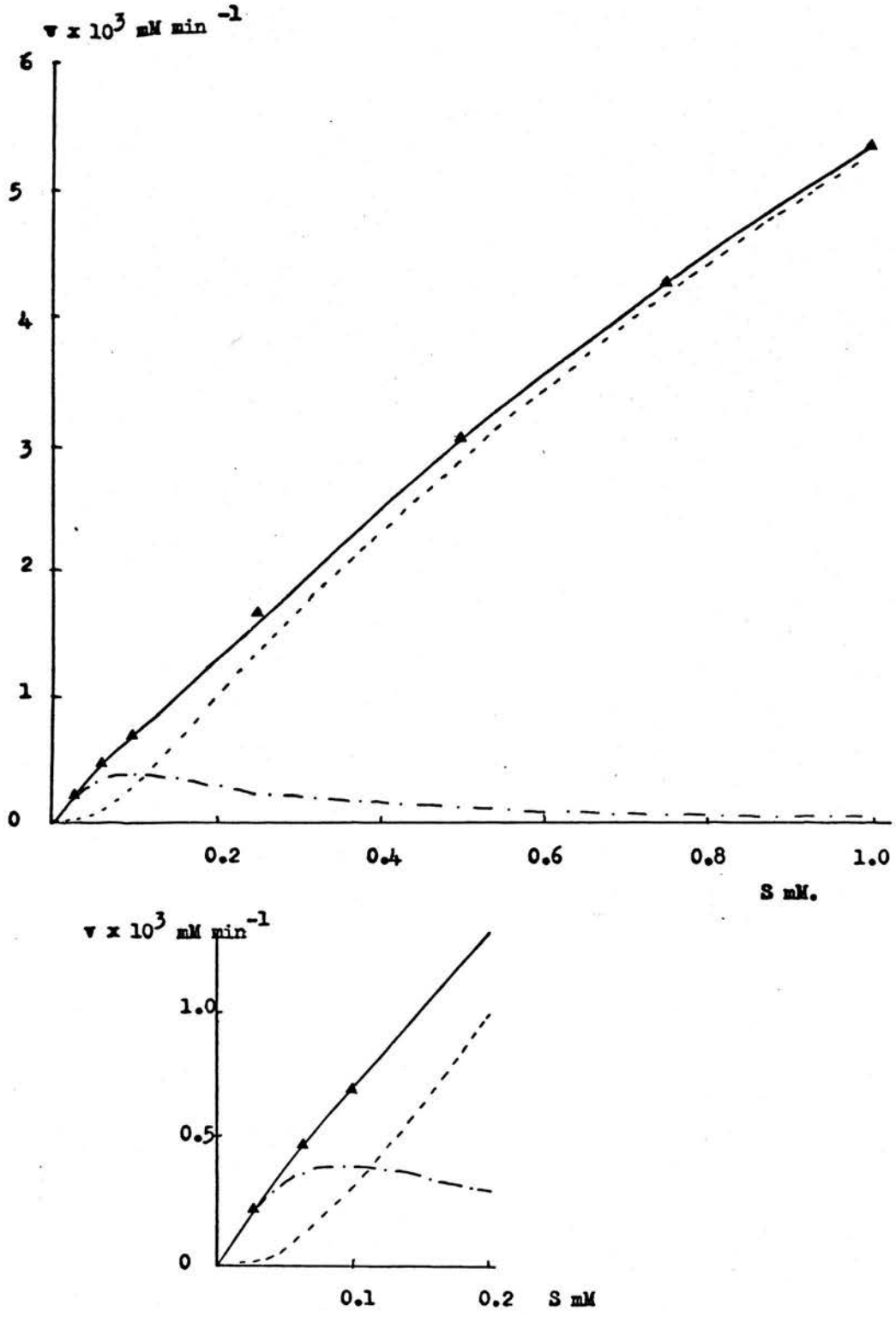


FIGURE 21 Resolution of a plot of v against S for the hydrolysis of Ac-Tyr-Leu-Val-His-methyl ester. Experimental points ▲ ; calculated line —— ;contribution from first term —.—.—; contribution from second term ------ (See equation opposite).

than V_1 and that K_2/K_3 should be greater than K_1. K_1 itself should be considerably above zero. At low substrate concentrations, i.e. as S approaches zero, the second term becomes negligible and the first term approaches $\frac{V_1 S}{K_1 + S}$. Classical kinetics are thus observed at low substrate concentrations and K_1 and V_1 may be estimated. At high substrate concentrations the first term becomes negligible and the second term approaches $\frac{V_2 S}{K_2/K_3 + S}$, hence an estimate of V_2 and $\frac{K_2}{K_3}$ can be found. Estimations of K_2 and K_3 have been obtained from the full equation, using the value of the initial velocity at a particular substrate concentration.

Values have been obtained for the peptide ac-Tyr-Leu-Val-His-methyl ester and the data has been fitted to the equation. Quite a good fit has been obtained as shown in figure 21, which also shows the resolution of the curve into its component terms.

The values of the constants are 21.9 and 209 μmole ml^{-1} min^{-1} x 10^{-4} for V_1 and V_2; 0.21, 0.113 mM for K_1 and K_2, and 0.0422 for K_3.

Sufficient observations have not been made to obtain good fit of the data to the equation with ac-Tyr-Leu-Val-His-amide.

Substrate activation has been reported for some proteases, notably papain and bromelain (e.g. Wharton et al. 1974) neither of which are closely related to the pepsins. Recently however, activation of pepsin towards small peptide substrates has been shown to take place using noncleavable but substrate-like molecules. For example the activators often included a large hydrophobic residue, but no

susceptible bond (Wang & Hofmann 1976b).

It is possible that the activators, whether they are substrate molecules or non-susceptible peptide molecules interact with the proposed secondary binding sites on the surface of the enzyme. A change in the conformation may take place, which can cause acceleration of the hydrolysis at the active site. Evidence from circular dichroism spectra for a conformational change in the enzyme penicillopepsin when an activator molecule became bound was reported by Wang et al.(1974).

In the light of the experiments reported by Wang and Hofmann (1976b), an experiment was performed to see whether the peptide ac-Ala-Leu-Val-His-methyl ester (which is barely hydrolysed by pepsin C) would activate pepsinCin the hydrolysis of ac-Tyr-Leu-Val-His-methyl ester in the low substrate concentration range (section 6.9). No measurable effect on the rate of hydrolysis was observed, thus this peptide does not activate the enzyme to the extent which is observed for a similar excess concentration of substrate. Apparently no inhibition took place either. It is possible that exactly opposing inhibition and activation processes were taking place. Further experiments varying the amount of added peptide would have to be done to ascertain this point.

The evidence from previously described work and from this project, on the effect of extending short peptides by one or more residues which results in increased rates of hydrolysis and from the activation at high substrate concentrations for certain peptides, suggests that binding sites distant from the catalytic site itself are important features of action of both pepsin A and pepsin C.

The use of small peptides can reveal aspects of the mechanism of action of pepsin on whole protein which might not be apparent from studies on whole proteins themselves. At first, the small synthetic substrates were used to test for specificity of the bond cleavage in peptic hydrolysis, but this has led on to mechanistic proposals of different kinds for the catalytic process and also to more general theories of peptic hydrolysis, as was discussed in the introduction.

The existence of more than one pepsin in man, pig, and other species has been recognised for many years. Whether any particular advantage is conferred on the animal by this mixture of enzymes is not clear. It has been shown (see introduction) that human pepsin A and gastricsin are homologous and also that bovine chymosin is homologous with pepsin from the species. It is likely that pepsins within other species will show homology to each other. Despite these homologies the enzymes do exhibit slightly different activities, both in the specificity of bond cleavage and the rate of hydrolysis of peptide bonds. They also show different stabilities at neutral or slightly alkaline values of pH. This type of variability is similar to that which has been found between the major pepsins from different species, for example pig and chicken.

It seems likely that during evolution a gene doubling took place before the major animal species diversion. The similarities which have been shown for pepsin C from man and pig and also for the major pepsin, support this, although pepsin C has not been demonstrated in all species investigated. Clearly more sequence studies would have to be done before the pattern of acid protease evolution can be fully appreciated.

Despite the clearly demonstrated existence of more than one pepsin-like enzyme in normal human gastric juice, having different activities, the enzymic activity is often collectively described as 'peptic activity'. Very little work has been done on the relative levels of the different pepsins normally found at different stages of development or in the levels found in gastric diseases. Although the physiology and endocrinology of gastric secretion has been studied, little is known about the control of pepsin production itself, whether it is directly related to the number of cells of origin or the number of copies of the various genes and so on.

Whilst it is beyond the scope of this thesis to discuss in detail the physiological occurrence and activity of pepsins, some consideration might be made of the value of measuring the various levels of the different pepsins in normal and a particular clinical condition, that of the peptic ulcer. The cause of ulceration is not known, but it is thought that both pepsin and acid are partly responsible (Magee 1974). Normally treatment is by the administration of an antacid, which raises the pH of the stomach and helps reduce pain, also abolishing peptic activity. More drastic action is by the surgical removal of the acid secreting cells. Whilst some attempt (Whitecross et al. 1974) has been made to compare the pattern of enzyme content in the gastric juice of patients with gastric ulcers, with that of healthy subjects, using electrophoretic techniques, little attempt has yet been made to quantify the individual enzymes present. The electrophoretic study showed no apparent difference in the enzymes present, between patients and most healthy subjects, although a slightly different pattern emerged for one of the healthy subjects. Sebus and Charbon (1964)

and Venables (1969) suggest that gastric ulceration may be associated with high peptic activity and have studied the problem using immunological techniques. They find that Group I pepsins (by the nomenclature of Samloff 1969) may contribute to the high activity. However, Group I consists of five electrophoretically separable components including zymogens and enzymes, from the chief mucous and neck cells, and hence unless more specific antibodies are raised to the individual components, this method is not very specific. Gastricsin is thought to be contained in Group II.

The assay system using synthetic peptides to measure the relative concentrations on activities of pepsin and gastricsin in a mixture may be applicable to this case. A system has been developed to allow the assay of each enzyme in a mixture of the pure enzymes and it is hoped that this can be successfully applied to measuring the quantities of the enzyme in gastric juice. Some purification will be necessary to remove low molecular weight material which interferes with the assay system, but it is likely that a short gel filtration step will be sufficient. An assay system is useful only if it is known exactly what is being assayed, and so it must be determined exactly what is present in the sample for analysis and what its contribution, or potential contribution to the measured activity is. Thus conversion of all zymogens to their enzyme before performing an assay is a useful step. Further tests should be done on the gastric juice to try to identify any other pepsin-like enzymes and to measure their activity, perhaps also with a synthetic substrate. Basal rather than stimulated secretions would be preferable.

The multiplicity of bands which are found on gel electrophoresis of gastric juice by different workers suggest that more than two enzymes exist, although they have not been isolated or characterised. Some of the bands may represent zymogens which have not been activated, or enzyme inhibitor complexes. This would rather depend on the activity of the gastric juice.

An advantage of the suggested assay system is that it should be fairly easy to automate, an important consideration for any system which might be used on a regular basis. The differential assay of Tang (1970) involves the haemoglobin assay which requires not only overnight dialysis for the preparation of the reagent, but also a precipitation and filtering stage. The general similarity between the two synthetic substrate assays may also be a useful feature.

The results presented in this thesis add to the evidence discussed elsewhere for the similarities between porcine pepsin C and pepsin A, by the kinetic hydrolysis of certain synthetic substrates. The pKa values of two catalytically important groups are similar for the two enzymes and the effect of activation by small peptides, substrates in the case of pepsin C is comparable to that observed for non-substrate molecules by Wang and Hofmann (1976). The difference in specificity pepsin C and pepsin A show towards peptides where a tyrosyl residue contributes the carbonyl group has been emphasised. This has been used in the setting up of a differential assay for the human enzymes, which show similar specificities, but in general lower activities towards the synthetic substrates.

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